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VOLUME XX DECEMBER, 1928 No. 4

RESEARCHES ON THE INTESTINAL PROTOZOA OF MONKEYS AND MAN.

I. GENERAL INTRODUCTION,

AND

II. DESCRIPTION OF THE WHOLE LIFE-HISTORY OF *ENTAMOEBA HISTOLYTICA* IN CULTURES.

BY CLIFFORD DOBELL, F.R.S.

National Institute for Medical Research, London, N.W. 3.

(With Plates XXII–XXV and 2 Text-figures.)

In magnis et voluisse sat est.—*Propertius*, Lib. II, x. 6.

I. GENERAL INTRODUCTION.

As the following paper forms a first instalment of a future series, in which I hope to record the results of researches which have now been in progress for several years, it seems to me advisable to explain the general scope and aims of this work—as a whole—before publishing any separate details. Accordingly, this introduction is to be regarded not merely as a preface to the paper which follows immediately, but as a general prolegomenon to a bigger work which is yet unwritten and in large part still undone.

My previous studies[1] of the intestinal protozoa of man taught me that there are many serious gaps in our knowledge of these organisms; but they also convinced me that most of them could be filled in by means of properly planned and carefully conducted experiments. Unfortunately, however, such experiments—to be conclusive—would usually have to be made on human beings, and some would be of such a character as to render this impracticable. Many people, doubtless, would be willing to submit themselves to irksome experiment for the sake of advancing knowledge; but probably few would volunteer —even were the acceptance of such an offer ethically admissible—for *post-mortem* examination at the conclusion. Yet this would often be essential, in order to obtain a decisive result. Consequently, the necessary experiments cannot at present be made in a way which is, from a scientific standpoint, entirely satisfactory.

But it has long seemed to me that much light could be shed on the intestinal protozoa of man by careful study of the intestinal protozoa of monkeys. It is already well known that monkeys, of many different species, harbour a varied

[1] See Dobell (1919) and Dobell and O'Connor (1921), where references to all my earlier publications will be found.

protozoal fauna remarkably similar to that characteristic of human beings; while monkeys as a group (if we except the Lemuroids and Marmosets) are sufficiently akin to us to warrant the assumption—with appropriate reservations—that what is generally true of their protozoal inhabitants is probably also true of ours. A concrete example will, perhaps, make my meaning clear and show the possibilities implicit in the researches which I contemplated.

An *Entamoeba* morphologically very like *E. histolytica* (of man) has been described—and, of course, variously named—as an intestinal parasite of several different monkeys: and this parasite (or, at least, some of the forms recorded) is said to cause both amoebic dysentery and hepatic abscess in anthropoids just as *E. histolytica* does in man. The alleged resemblances between the human and the simian parasites are, in fact, so close, and the points of difference so few and doubtful, that when I attempted some years ago to make a revision of the amoebae living in man[1] I was unable to discriminate between the parasites of these different hosts with any certainty. But I was also unable, from the data then available, to assign any of the amoebae described from monkeys to the species *E. histolytica*. Does this species itself occur naturally in *any* species of monkey? How many species of monkeys are infected with these *histolytica*-like amoebae? Are the closely similar forms found in different monkeys all of one species? If they are different, in what ways do they differ, and how can they be distinguished from one another and from the parasite of man? How do these simian forms live in their natural hosts, and how are they able to cause dysentery and hepatic abscesses in them? To these and many other similar questions no satisfactory answers have hitherto been found.

Yet it is certain that correct answers to all such inquiries will have to be obtained—sooner or later—if we are to know all about *E. histolytica* and its activities. Whatever the answers may ultimately prove to be, they will supply us with most valuable information: and if it should be found that any species of monkey is naturally infected with *E. histolytica* itself, then we should have at our immediate disposal an animal which could replace man for experimental purposes, and which would therefore offer us almost unlimited opportunities for extending our knowledge of the human parasite. When it is realized that the same can be said (*mutatis mutandis*) of nearly all the other intestinal protozoa of man—not merely of *E. histolytica*—then I think it must be admitted that the intestinal protozoa of monkeys offer us a most promising and attractive field for investigation.

These and many other cognate considerations led me to undertake my present researches. I am well aware that the problems involved are vast, and that they will not all be solved in my lifetime. A critical survey of the relevant literature has already convinced me, moreover, that our present "knowledge" is little more than ignorance and guesswork. It abounds with manifest mal-observations and misdescriptions—zoological, physiological, and pathological —of "parasites" found in "the monkey" (whatever that may be supposed to

[1] See Dobell (1919)—especially pp. 131–133.

denote); for, with rare exceptions, the students of this subject appear to have been content with observations made upon inaccurately determined protozoa —often obtained from doubtfully identified hosts—and with experiments that are uncritical in the extreme. I need hardly cite specific instances: anybody familiar with the writings on this branch of protozoology will have little difficulty in calling to mind plenty of examples. But I may recall, as a glaring instance of the untrustworthy work to which I refer, a published account of the (alleged) transmission of amoebic dysentery from man to monkey. The author —who shall here be nameless—claims to have infected a monkey with *E. histolytica* experimentally. Yet it is clear, from his words, that he could not certainly distinguish this amoeba from the other species living in man: he was unaware that many monkeys are naturally infected with a closely similar organism: he never even examined his solitary ape in order to find out what it was infected with before the experiment, nor did he exactly determine its own species: and as evidence of success he finally figured—*inter alia*—what appears to be a flagellate (of a genus common in both men and monkeys) which he found in the dejecta of his experimental animal after inoculation. Observations such as these obviously add nothing to our knowledge. They merely create difficulties and unnecessary problems for future workers.

I am convinced that no advance is possible along similar lines. What we want is certain knowledge—however small in amount—derived from accurate observation and critical experiment. To my mind no absolutely certain zoological conclusions can be drawn from any observations yet published on any species of intestinal protozoon found in any species of monkey. Neither the morphology nor the life-history of a single one of these is known in full detail; and nobody is yet in a position to say whether any intestinal protozoon hitherto found in any monkey is, or is not, identical with the similar species known to occur in man. But the simian protozoa are obviously worthy of study for their own sake: and if their possible identity with the corresponding forms in man be considered, it seems to me equally obvious that they must possess a very real and urgent human interest.

Some few years ago I resolved to examine this subject, and I decided to begin by making an intensive study of the intestinal protozoa occurring naturally in a few individual monkeys, of accurately determined species; and I then proposed to compare these protozoa with those of man, and to study their relations to one another and to their respective hosts experimentally. As it was immaterial to me what particular monkeys I studied in this way, I took the first that were available. These happened to be Macaques (*Macacus rhesus* and *M. sinicus*)[1], which had been discarded by fellow-workers[2] in the Institute. They were all more or less normal animals—obtained originally from dealers, and

[1] Since the scientific nomenclature of the Primates is still in a state of flux, and as I am incompetent to form an opinion on such questions, I shall adopt, as a general rule, the names accepted by Forbes (1896, 1897) for the species whose protozoa I describe.

[2] For these animals I was indebted to Dr P. P. Laidlaw and Dr J. Mair, to whom I would here express my thanks once more.

of unknown earlier provenance (except in so far as their origin was deducible from their known geographical distribution[1])—which had never been inoculated with intestinal protozoa, or studied from a protozoological standpoint.

I began[2] with only four immature monkeys—three *M. sinicus* (two males and a female), and one *M. rhesus* (a male); but I have since been able to add a grown male of the former species, and a very young female of the latter, to their number. I have also been able to obtain, through the kindness of various colleagues, valuable material[3] for cultivation and for *post-mortem* examination from several other specimens of *M. rhesus* and from one *M. nemestrinus*.

At the outset I realized that it would be impossible to make any satisfactory experiments over a long period with untamed and unhealthy monkeys. I therefore devoted much time to taming my animals, and learning how to keep them fit in captivity—as I had had no previous experience in the management of monkeys. The time and trouble expended in this way have been well spent, however, as all my monkeys are now normal, healthy, and manageable; so that I can easily handle and treat them in any way necessary for parasitological investigation, and can obtain their co-operation in any experiments which I desire to perform. My own experience—though limited—has fully confirmed me in the belief that observations and experiments made upon untamed and unwilling captive monkeys have very little scientific value[4]. The Macaques are all tropical or subtropical in habitat, and gregarious in disposition; and they are naturally very active, mischievous, suspicious, and quarrelsome, and—by human standards—excessively and disgustingly dirty and unhygienic in their habits[5]. It is therefore not at all easy to keep them happy and healthy in confinement in a climate like that of England. They require constant attention, and are very difficult animals to employ for experimental purposes. They soon become unhealthy if they are unhappy; and they are never happy without proper food, exercise, air, light, warmth, and companionship. To anybody who has seen wild monkeys in their natural environment, the average macaque exhibited in zoological gardens or studied in laboratories is a distressing

[1] If a line be drawn across the Indian peninsula from Bombay on the west to the Godavari River on the east, then *M. sinicus* is confined to the southern section (not including Ceylon), while *M. rhesus* occupies the northern, together with a considerable part of the mainland eastwards. *M. sinicus* is thus the more tropical species, and is the more difficult to acclimatize to temperate conditions. *M. rhesus*—which may ascend to the snow-line in its natural state—is much hardier, and therefore easier to keep in England.

[2] I began my work in earnest—after making desultory observations and studying the literature for a good many years—in June, 1924.

[3] For this I am chiefly indebted to Dr J. Mair, Dr W. J. Purdy, Dr J. A. Campbell, and Sir Charles Ballance.

[4] As a very simple instance, I may note that even so trivial an operation as the taking of a monkey's temperature may easily miscarry: for the body-temperature of a normal macaque may rise several degrees merely as a result of fright and exertion, and the record of a temperature taken from a wild and struggling animal is therefore almost worthless.

[5] In the words of Gargantua "*Ce qu'il [le singe] faict est tout conchier et dégaster....*" (Rabelais, I. 40): and as a more recent writer has remarked—with equal felicity—"The monkey seems to spend its whole life in pursuing unworthy aims, and then eating them."

spectacle. It is usually a scared, morose, ill-conditioned, lousy, imperfectly grown, and often half-paralysed and always revoltingly filth-besmeared animal —so abnormal, indeed, as to be an almost unrecognizable specimen of its species. Experiments or observations made for pathological purposes upon such already highly pathological individuals are—to my mind—obviously futile.

I have now had nearly four years' daily experience with macaques, and it has convinced me that the secret of success in keeping these animals in confinement is *personal attention*. They must be kept as pets—not as savage beasts—and each individual's requirements must be studied and satisfied. I may be wrong: but my own monkeys are now much better specimens than any others I have seen in captivity, and I have not yet lost a single animal as a result of tuberculosis, pneumonia, "cage paralysis," or any other disease.

It might be urged, with some justice, that monkeys are unsuitable subjects for experimentation in a country like England. The conditions under which they must necessarily be kept are obviously so abnormal that all experiments are open to the objection of having been performed in an unnatural environment. It might even be said that monkeys—and their parasites—can only be studied properly in their native land. But this objection is, I think, invalid—so far as my own experimental macaques are concerned. These are now thoroughly acclimatized, and for all practical purposes "normal." They live and grow and exercise all their bodily functions in a normal manner, and are possibly in even better physical condition than they would have been in if they had remained in their native habitat. And there is, moreover, one great advantage—from the protozoological standpoint—in studying these animals in England rather than in India: they are here completely isolated from their fellows, and thus unable to acquire new infections from extraneous sources. We do not know—with absolute certainty—how, when, or where wild macaques get infected with their intestinal protozoa: but if they are kept in captivity in England, all possible sources of infection can be known and, if necessary, excluded. It therefore seems to me that it is even better—certainly not worse —to study the intestinal protozoa of monkeys in healthy captive animals in a temperate climate than in wild individuals on their native soil.

In order to carry out many of the experiments which I had planned, it was necessary, in the first place, to discover trustworthy methods of cultivating the intestinal protozoa of monkeys. With this object I undertook a series of researches with Dr P. P. Laidlaw—the results of which have now been, in part, published[1]. By modifying and improving the technique of Boeck and Drbohlav (1925)[2] we were able to devise methods and culture-media which have been of the greatest service to me in my main investigations. Without this preliminary work it would, indeed, have been impossible even to attempt some of the experiments which I have now brought to a successful conclusion. For example,

[1] See Dobell and Laidlaw (1926 *a*) and Dobell (1927).

[2] I refer here to their chief paper, but their results were actually made known earlier. I have noted the salient historical details elsewhere (Dobell, 1925).

our methods of cultivating some of the intestinal amoebae have already been brought to such a stage of (comparative) perfection that I can now obtain the whole life-cycle of these organisms *in vitro* at will—free forms, cysts, or any other stage desired: and I thus have at my disposal unlimited material, in any stage of development which I require, for experimental study. The importance of this, in research of this character, will be self-evident; and I would therefore once more express my gratitude to Dr Laidlaw for the very great assistance which his collaboration has afforded and still affords me in my work.

Although, as already indicated, my aim at present is to make a detailed study of the intestinal protozoa of a few monkeys—studying them especially with regard to their relations to the similar forms known to occur in man—it may not be without interest to give here a brief synopsis of the organisms which I have hitherto met with and investigated. All the intestinal protozoa which I have yet found in macaques are (to me) morphologically indistinguishable from the corresponding species found in man: and as my work has now convinced me that, in some species at least, this similarity is due to real specific identity, I shall use the names which appear to me to express the truth. I have found the following intestinal protozoa in the macaques named:

In **Macacus sinicus** (L.)—

Entamoeba histolytica (including strains with small cysts)
Entamoeba coli
Endolimax nana
Giardia sp.
Enteromonas sp.[1]

In **Macacus rhesus** (Audebert)—

Entamoeba histolytica (including strains with small cysts)
Entamoeba coli
Endolimax nana
Giardia sp.
Trichomonas sp.
Chilomastix sp.
Enteromonas sp.
Balantidium coli

In **Macacus nemestrinus** (L.)—

Entamoeba histolytica (including a strain with small cysts)
Entamoeba coli
Endolimax nana
Trichomonas sp.
Enteromonas sp.

I have cultivated all these species of protozoa, at various times and for various periods, with the exception of *Giardia* and *Balantidium*[2]; and with most of them I have already performed numerous experiments. Some of these are decisive, others are inconclusive, others are still in progress. In the course of this work all manner of side-issues have presented themselves, and have required investigation. Moreover, while studying the simian species of protozoa I have continually had to restudy and compare anew the corresponding human

[1] Morphologically indistinguishable from *E. hominis* (Da Fonseca) Dobell et O'Connor = *Tricercomonas intestinalis* Wenyon et O'Connor. (See Dobell and O'Connor (1921) p. 80.)

[2] *Balantidium coli* (from the pig) has recently been cultivated successfully, by our methods, by Jameson (1927). I have no doubt that the forms from *M. rhesus* could be similarly cultivated, but I have not yet attempted it. None of my own monkeys is infected with this ciliate.

forms, and repeat my experiments with these. Consequently, some of my researches have become long and complicated, and their final outcome is still uncertain. But other experiments are now finished, and some of my findings are already sufficiently definite to warrant publication. I propose, therefore, to publish my results piecemeal—as I obtain them, and can find time to write them up—without regard to their ultimate connexion with one another. I shall publish them, not in the logical sequence which they would properly assume if the whole work were done, but in the order in which the various parts are completed. This method of publication has obvious disadvantages, but it has the advantage that it will make my results known to other workers much earlier than they would be if I were to wait until every detail had been fully worked out. It will still require several years, at least, before I can finish some of the experiments upon which I have already embarked; and as I continually find myself forced to begin new ones, the end of these researches, in their entirety, is not yet in sight.

A few of my results have already been recorded incidentally in the annual *Reports* of the Medical Research Council, and it may be of interest to other workers along similar lines if I here note the chief points mentioned in these publications. In the *Report* for 1924–25 it was recorded (pp. 31, 32) that I had then succeeded, in collaboration with Dr Laidlaw, in cultivating several of the intestinal amoebae and flagellates of macaques and man by new modifications of the method of Boeck and Drbohlav; and further reference to this work was made in the *Report* for 1925–26 (p. 35). A fuller account of these researches has since appeared (Dobell and Laidlaw, 1926 *a*). In the first *Report* (1924–25) it was also noted that I had been able to infect kittens experimentally with "*E. histolytica*" of simian origin, and had studied the amoebic dysentery thus produced in these animals. This study has been continued, and I hope to publish a detailed description of my findings at an early date.

In the *Reports* for 1924–25 and for 1925–26 it was mentioned also that Dr Laidlaw and I had investigated the action of emetine and various other chemicals on cultures of *E. histolytica* and other entozoic amoebae of man and monkeys. This work was more fully described by us later (Dobell and Laidlaw, 1926), and is being continued.

In the *Report* for 1925–26 it was further stated (p. 36) that I had been able to cultivate *Endolimax nana*[1] from man, and the corresponding forms from *M. rhesus* and *M. sinicus*; and that I had succeeded in transmitting the *Endolimax* of *M. sinicus* from this monkey to man, and from man to *M. rhesus*. A complete account of these researches I hope to be able to publish shortly in the present series.

In the current *Report* (1926–27) it is recorded—*inter alia*—that I have been able to transmit *E. histolytica* from man to *M. sinicus*; and that I have found—in collaboration with Miss A. Bishop—that natural infections with

[1] The whole life-history of this species—including encystation and excystation—and also that of *Entamoeba coli*, was obtained *in vitro*. Cf. Dobell and Laidlaw (1926 *a*).

E. histolytica in macaques are radically curable by means of emetine. Detailed descriptions of these researches will be published as soon as possible.

The annual *Report* for 1924–25 contained a statement that Dr Laidlaw and I had been able, "with *E. histolytica* from man" and "with two species of amoeba from monkeys," "to obtain the complete life-cycle in cultivation outside the body for the first time"; and it was there recorded (p. 32) that in the course of our work "the active amoebae have been cultivated up to the final stage of encystation; and by hatching the cysts so formed, new cultures have been obtained repeatedly. The conditions necessary for encystation and excystation have been specially studied." Dr Laidlaw and I published[1] later a fuller account of our work, and I have elsewhere[2] discussed various experimental details. The following paper embodies the results of my own particular study of the life-cycle of *E. histolytica* in cultures made by our methods.

If any reader should wonder at the length of time that has elapsed between our first announcement and the publication of the ensuing paper, I would ask him to consider the amount of labour necessary to investigate this subject properly: though nobody who has not himself attempted to unravel this tangled problem conscientiously *de novo* can have anything but a faint conception of the time and work—not to mention patience and ingenuity—really requisite. I hope the following instalment—with those to appear subsequently —will constitute its own justification.

In conclusion, I beg all fellow-workers to send me copies of their own publications, or to notify me of their published results—especially if these should appear in some out-of-the-way journal. I hope eventually to publish a full bibliography of the works dealing with the intestinal protozoa of monkeys, and I shall therefore be most grateful for any assistance in its compilation.

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Idem, for 1925–26. *Ibid.*, 1926. [Published Feb. 1927.] Pp. 35, 36.

Idem, for 1926–27. *Ibid.*, 1928. [Published in Jan.] Pp. 33–35.

[1] Dobell and Laidlaw (1926 *a*).

[2] Dobell (1927).

II. DESCRIPTION OF THE WHOLE LIFE-HISTORY OF *ENTAMOEBA HISTOLYTICA* IN CULTURES

Door Arbeijt en Naarstigheijt komt men tot saaken die men te vooren onna speurlijk agten.
—*Antoni van Leeuwenhoek* (1698).

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I. INTRODUCTORY.

ALTHOUGH the life-history of *Entamoeba histolytica* has now been known, in broad outline, for some years, there is still much uncertainty regarding various important details. The active amoebae, as they occur in the bodily lesions or in the stools of man, and the cysts and all stages of their formation in the bowel, have long been familiar. It has long been known, also, that when the cysts are swallowed—by a man or other infectible animal—they somehow give rise to an infection with active amoebae once more. But exactly how these relatively large uninucleate amoeboid organisms are formed from the small quadrinucleate cysts has never yet been determined.

Only a few years ago it appeared to me almost hopeless to attempt to discover these missing stages; for normally they occur in an inaccessible part of the body of man, and man is not an animal available for fatal experiment. Moreover, all experiments attempted with other hosts had failed. But the discoveries of Boeck and Drbohlav opened up new lines of inquiry, and suggested to me the possibility of solving the problem indirectly. These workers demonstrated[1] that *E. histolytica* could be cultivated outside the human body; and one of their observations indicated that the amoebae might encyst[2], while another showed that they might also excyst[3], *in vitro*. On repeating and varying their experiments, Dr Laidlaw and I found that encystation could,

[1] Boeck and Drbohlav (1925).
[2] *Ibid.*, p. 393.
[3] *Ibid.*, p. 385.

indeed, be induced in cultures; and that new cultures could then be obtained by incubating the resultant cysts[1].

Our first successful attempt to obtain the complete life-cycle of *E. histolytica* outside the human body was made in December, 1924. At the very beginning of this month, Dr Laidlaw discovered that by adding rice-starch to ordinary Boeck-Drbohlav cultures much richer growths of amoebae could be obtained: and our further study of such cultures soon showed us that these starch-fed organisms finally formed numerous typical 4-nucleate cysts. These cysts generally died on continued incubation, but we found that they could remain alive for many days at room-temperature.

In this manner we were then able to induce encystation *in vitro* in three different strains of *E. histolytica*—one (*D.*) derived from man, the other two (*M.*4 and *J.*) from *Macacus rhesus*. On 7 December 1924, I inoculated some of these culture-cysts—which I had kept in distilled water for two days at room-temperature—into fresh medium and returned them to the incubator. On the following day I found that they had (apparently) hatched and produced an excellent culture of amoebae. To make certain that these were really derived from the cysts, and not from free forms out of the original culture (which might conceivably have survived along with them), I repeated the experiment on 10. XII. 24 with cysts which I had kept at room-temperature for nine days—feeling sure that no amoeba of this species could possibly live at this temperature for so long. The result was identical.

It was thus clear that we had found a method of obtaining the complete life-cycle of *E. histolytica* apart from its host; since we had obtained cysts from amoebae, and amoebae again from these cysts. Consequently, it only remained to repeat these experiments, with greater precautions, and study all stages in detail. Nevertheless, this proved to be one of the hardest tasks I have ever tried to perform, and its performance has required over three years of unbroken work.

Although I immediately cultivated and studied numerous strains of *E. histolytica*, and experimented continuously with the aim of defining and eliminating all sources of error, it was not until the autumn of 1926 that I succeeded in obtaining a strain (*K.* 28 c) which fulfilled all my requirements. Scores of other strains which I isolated were all unsatisfactory in some particular, and as a rule I could not induce them to encyst regularly. Upon Strain *K.* 28 c, therefore, I concentrated my attention; though I did not cease to study the amoebae in their original host, and in other animals which I infected

[1] Dobell and Laidlaw (1926 *a*). These results were first announced in the annual *Report of the Medical Research Council* for 1924–25 (published in January, 1926). At the time when our first successful experiments were made (December, 1924) the work of Boeck and Drbohlav (1925) had not been published. But I learnt of their results from Dr Drbohlav personally in November, 1924; and in January, 1925, I received from Dr Boeck a MS. copy of their unpublished paper. I add these details lest it might appear—from the dates recorded—that my observations preceded those of Boeck and Drbohlav. Their joint work was actually finished, and its main results known to me, before mine began.

experimentally. At the same time I continued to study many other strains of amoebae—of the same and different species, from men and monkeys—so that I have had much additional material for comparison.

Some of the statements made in the present paper are founded upon observations or experiments forming part of other researches still in progress. These will, I hope, be reported before long. In the meantime, I must beg the reader's indulgence if I sometimes appear to give conclusions without adequate evidence. Such evidence is not lacking, nor withheld through want of frankness on my part or with a desire to cover up defects in my work, but because I intend to publish it later in another connexion.

As I have carried out my own researches regardless of the work of others, I shall present my findings first, and shall consider previously published results in the final discussion[1]. I am aware that by thus inverting the historic sequence I lay myself open to criticism, but I am also convinced that the history of the present subject cannot be profitably discussed before the facts are established.

II. MATERIAL AND METHODS.

To study the life-history of *E. histolytica* properly *in vitro*, it is obviously first necessary to devise suitable methods of cultivation. Boeck and Drbohlav discovered a method in 1924[2], and later Dr Laidlaw and I were able to improve it[3]. The methods which I have used are those which we have already described. As previously noted, I have now cultivated a large number of amoebae (from Man, *Macacus rhesus*, *M. sinicus*, *M. nemestrinus*, and experimentally infected kittens), but hitherto I have been able to study exhaustively only a single strain of *E. histolytica*—the one (*K.* 28 c) which ultimately proved most suitable for my purpose.

In a study of this sort, it is clearly not legitimate merely to ascertain that a given man or monkey is infected with *E. histolytica*, and then—without further evidence—to assign to this species all or any of the entamoebae cultivated from the faeces of this particular host. Other species grow readily in the culture-media necessarily employed; and to make sure that any culture is specifically and racially pure is not easy. Elaborate precautions must, indeed, be taken if one wishes to obtain unquestionable material for study. Consequently, it is of prime importance to know the exact source of any strain specially investigated, and the particular evidence for its identity and its purity.

Origin of Strain K. 28 c.—This strain of *E. histolytica* was originally derived from an adult male specimen of *Macacus sinicus*. I first examined the faeces of this monkey (*B*, then belonging to Dr J. Mair) on 13 August 1924, and found him infected with *E. histolytica*. He came into my possession later—at the beginning of 1925—and was afterwards under my constant personal observation until I killed him in September 1927. During this period he never suffered

[1] See p. 401, *infra*.

[2] See Boeck and Drbohlav (1925) for their fullest account.

[3] Dobell and Laidlaw (1926 *a*).

from dysentery or any other intestinal disorder. From March 1925 until September 1926 (18 months) I examined his faeces exhaustively, both microscopically and culturally, on 33 occasions. I studied and cultivated all the amoebae I could find in him, and performed numerous experiments with them. As a result of all my work I found that he was infected with *E. histolytica* (two strains at least—one with cysts 12–14 μ in diameter, the other with cysts of 7–8 μ), and with *Endolimax nana*. No other species of amoeba was ever discovered, either culturally or by direct microscopic examination[1]. The evidence that this animal was infected with these two species only is therefore very strong.

On 12 September 1926 I examined this monkey's faeces, and found—as usual—abundant cysts of *E. histolytica*[2] (the large strain, with cysts 12–14 μ in diameter). These cysts were washed and concentrated in sterile distilled water; then treated with 0·2 per cent. hydrochloric acid for 1½ hours at room-temperature, to free them from concomitant amoebae[3]; again examined, and a small sample inoculated into a culture-tube; and finally fed to a healthy kitten (No. 28)—five hours after leaving the monkey's intestine. Kitten 28 was six weeks old at the time of the experiment. He was born in my animal-room at the Institute, had been under my daily personal observation since birth, and had never been used previously for any other experiments.

The culture inoculated with cysts produced, as usual, a good crop of normal *E. histolytica* on incubation. After the experimental feeding, Kitten 28 remained normal until 18 September (six days): but on this date it passed some bloody mucus with its faeces—the beginning of a typical attack of acute amoebic dysentery. In the blood and mucus I found typical active forms of *E. histolytica*, which I was able to cultivate without difficulty. In one such culture—made in horse-serum and Ringer-eggwhite medium with rice-starch[4]—a very fine growth of amoebae was obtained. At first ordinary large active forms of *E. histolytica* alone were present, but after two days' incubation numerous cysts appeared. Careful microscopic examination showed that these were all, without exception, typical cysts of *E. histolytica* (approximately 13·5 μ in mean diameter). No cysts of *E. coli*, or of the small strain of *E. histolytica*, or of *Endolimax nana*, were discoverable after exhaustive search. I kept some of these cysts at room-temperature for six days[5], and then inoculated them into fresh medium. On incubation they hatched normally, and

[1] The faeces of this animal were also many times examined from September 1926 until September 1927—always with the same result; but the examinations made after 12. IX. 26, though confirmatory, have no direct bearing upon the experiments about to be described.—Actually I examined this monkey's faeces more frequently than is indicated above—the 33 examinations mentioned being those which were made thoroughly, and of which the findings were recorded in full.

[2] Cysts of *E. nana*, with which this monkey was known to be infected, were also present; but no small cysts of *E. histolytica* could be found in this sample, or in the concentrate.

[3] See Dobell (1927).

[4] See Dobell and Laidlaw (1926 *a*).

[5] To kill all accompanying amoebae (see Dobell, 1927).

produced a fine crop of amoebae which have been subcultivated ever since as Strain *K.* 28 c.

Since I first isolated this strain in 1926 it has never left my hands. It is now, at the moment of writing, at its 220th serial subculture, and has been under cultivation for 20 months. Morphologically it is a typical *E. histolytica*, showing no admixture of other species. It forms, in cultures, typical 4-nucleate cysts with great regularity; and these cysts, when measured and counted, invariably give a unimodal curve of the same character. Their mean diameter is always approximately 13·5 μ. On cultivation Strain *K.* 28 c—as I determined experimentally—soon lost its infectivity for kittens[1]; but it remained infective for *M. sinicus* for at least seven months[2], and like all other strains tested it has been found to be extremely sensitive to emetine *in vitro*[3].

From its history and characteristics I am satisfied that Strain *K.* 28 c is specifically pure. It was derived originally from a thoroughly studied host, which was almost certainly uninfected with any similar species. It was then conveyed, by means of carefully controlled cysts, to an untainted cat, in which it produced typical symptoms and from which it was recovered in pure culture. If *E. coli* had accompanied this strain in its original simian host, it would certainly have been eliminated by passage through a kitten; for I have never—after repeated trial[4]—succeeded in infecting kittens with this species (from man or *M. sinicus*). The original host was known to be also infected with a small strain of *E. histolytica* and with *E. nana*; but I have likewise attempted to infect kittens with both these organisms (isolated in pure culture from *M. sinicus*) and have invariably failed[5]. It is thus highly probable that if a kitten were fed on a mixture of cysts (of all these species) it could acquire infection with one species only—*E. histolytica*, the typical form with "large" cysts (12–14 μ). At all events, in the present case only this one species was recovered from the experimentally infected kitten, and cysts of this species alone were subsequently produced in the primary culture. From these cysts Strain *K.* 28 c was started, and their descendants answer all the requirements of a single species and a pure race of that species. In every respect *K.* 28 c thus appears to me above suspicion as a pure strain of *E. histolytica*.

The only obvious objection which might be raised against this conclusion is, I think, that we have as yet no proof that the amoeba which I call "*E. histolytica*" in *Macacus sinicus* is specifically identical with the similar form—properly so called—in man. But that this is so I am now convinced, as a result of many observations and experiments. I hope to publish these shortly, but

[1] A human strain, previously described, behaved in the same way (Dobell and Laidlaw, 1926 *a*). The animal experiments alluded to here will be published in detail later.

[2] I have so far been able to test this strain only this once, when I infected a clean *M. sinicus* by feeding it on culture-cysts. (Unpublished observations.)

[3] See Dobell and Laidlaw (1926). The action of emetine on Strain *K.* 28 c has been carefully studied (Laidlaw, Dobell, and Bishop, 1928).

[4] Unpublished observations.

[5] These experiments are unpublished, and will be described in detail later.

meantime must ask the reader to accept this conclusion on insufficient evidence. I will only say here that all the various strains of "*E. histolytica*" which I have yet studied—whether derived originally from Man, *M. sinicus*, *M. rhesus*, or *M. nemestrinus*—are morphologically identical, in all essential specific characters, and develop in the same way in cultures. I have devoted particular attention to Strain *K.* 28 c (from *M. sinicus*), but I have also studied two other pure strains in considerable detail. One of these (*DKB.*) was originally derived from a man; the other (*DMK.*) is a human strain which was recovered from a monkey (*M. sinicus*) experimentally infected with a human strain of the same origin. Both these strains can be readily cultivated and induced to encyst and excyst by the methods successfully used with *K.* 28 c, and all three strains develop in cultures in the same way.

When I say that Strain *K.* 28 c is "pure," I refer of course to the amoebae. All cultures are really "impure," in the sense that they consist of amoebae and a mixed bacterial flora—on which, in part, the amoebae feed. In none of my cultures was this flora completely known. Every strain of amoebae isolated from faeces in the customary manner probably contains a different mixture of bacteria: but when once a strain is established, the mixture of micro-organisms reaches a stable equilibrium, and, if care be taken to avoid the introduction of other bacteria, it remains constant. The flora thus becomes an invariable factor in all such cultures, though its exact composition may be unknown. It has been shown elsewhere[1] that the accompanying bacteria often exercise a profound influence upon the growth and development of any particular strain of amoebae: and it is therefore absolutely necessary to keep every "pure" strain—if used for experiment—in the same bacterial environment throughout. Only by observing this precaution rigidly can unvarying and certainly predictable results be secured.

In order to make the desired observations on Strain *K.* 28 c, after I had established it in "pure" culture, it was necessary to discover some means of inducing encystation and then excystation *in vitro*—not occasionally and irregularly, but regularly and at will. This I accomplished—after innumerable heartbreaking experiments with other strains[2]—and I will therefore now describe the one method which ultimately succeeded.

Methods used for inducing Encystation and Excystation in vitro.—It may be recalled that Dr Laidlaw and I found originally[3] that several strains of *E. histolytica* could be made to encyst by adding rice-starch to the culture-medium. We found, however, that when we continued to cultivate these strains in this way they gradually ceased to form cysts: and we failed to obtain a strain of amoebae which would encyst regularly and abundantly, and to determine more exactly the factors which induce encystation.

Since many of our strains encysted excellently when first grown in media

[1] Dobell and Laidlaw (1926 *a*).

[2] A few of these experiments have already been described (Dobell and Laidlaw, 1926 *a*).

[3] See Dobell and Laidlaw (1926 *a*).

containing starch, but ceased to encyst when fed continuously on this carbohydrate, it occurred to me that a strain, if kept in starch-free medium (in which encystation does not usually take place), might possibly encyst whenever starch was first offered to it. I therefore tried the experiment. With Strain *K*. 28 c—and with several others, though not with all—this procedure proved entirely successful.

After hatching the cysts from which this strain was derived, I subcultivated it in the starch-free medium[1] of Boeck and Drbohlav (1925). In this, originally, it grew well, but required transplantation every day. Later it grew even better, and the cultures lived for two or three days. The improvement continued, moreover, so that at present it is only necessary to make subcultures every four or five days, while they often live for a week. Excellent cultures, containing numerous amoebae but only very rare cysts, can now be readily made and propagated in this way.

Whenever I desire to obtain cysts, I inoculate these amoebae into a tube of similar culture-medium to which I have previously added a little solid rice-starch. Such a culture invariably undergoes encystation—usually producing the maximum number of mature 4-nucleate cysts after incubation at 37° C. for 48 hours. In any shorter time it contains abundant active amoebae, or precystic forms and immature cysts; and on more prolonged incubation—up to a week or so—it generally contains amoebae, in decreasing numbers, and few or no cysts. By taking a culture from the incubator at the appropriate time, therefore, I can easily obtain amoebae, ripe cysts, or any intermediate stage desired.

Throughout the whole period during which this strain has been under cultivation, it has never failed to respond in the way just described. But to insure success it is necessary to pay great attention to details. The composition of the bacterial flora must not be altered. The culture-medium must therefore be properly prepared, and sterile before inoculation: the added rice-starch must also be sterile. Extraneous micro-organisms—if introduced accidentally—usually frustrate the experiment. Moreover, the temperature must not vary, but must be maintained continuously at 36·5°–38° C. Incubation at temperatures above or below, or at varying temperatures, gives uncertain results. Provided that the precautions just indicated are observed, encystation occurs with the regularity of clockwork. In the last 20 months I have performed this experiment more than 90 times, and—barring one or two accidents due to contaminated media or incubator-trouble—I have never had a failure. I can always obtain cysts in any quantity at any time.

Although I now usually obtain cysts in the manner just described, many other media—provided that they contain rice-starch and the same bacterial flora—have been found equally suitable for inducing encystation. Inspissated

[1] The medium which I use is made from coagulated whole eggs emulsified in Ringer's fluid, and covered with dilute horse-serum. It is that called "Ehs" in a former publication (Dobell and Laidlaw, 1926 *a*).

horse-serum with Ringer-eggwhite and starch works just as well, as do various other combinations of egg and serum[1]. I have even obtained good crops of cysts by inoculating the amoebae into tubes made from ordinary slopes of nutrient agar covered with distilled water, to which a little rice-starch was added[2]; and also in liquid cultures consisting of nothing but dilute horse-serum (one part to eight parts of Ringer's fluid) and starch. Any medium in which the amoebae can live and grow, provided that it is supplemented with rice-starch[3], appears to suffice if the amoebae have been grown previously in starch-free medium.

It is thus extremely easy to obtain all stages in the life-history of Strain *K.* 28 c up to the point at which encystment is complete. The method is simplicity itself. To obtain the later stages—excystation, and development to the adult uninucleate amoeboid form—is equally easy. It is only necessary to transfer mature living cysts to fresh medium and to incubate them at body-temperature[4]. If this is done—provided that no foreign bacteria are introduced—they hatch readily, and within 24 hours produce a culture of typical amoebae once more. These can then be cultivated in the usual ways, and again made to encyst by employing the same methods; and the process can be repeated *ad libitum.*

But although it is thus almost childishly simple—now that the method has been discovered—to obtain cysts from amoebae, and amoebae again from cysts, it is far from easy to study this development in every detail. It is, in fact, extremely difficult and tedious. Much the most difficult stages to study are those immediately following excystation; and in order to avoid all pitfalls in obtaining and observing these stages, great caution must be exercised. As I have already considered the chief sources of error and their remedies in a previous paper[5], I can now deal with these summarily.

I have shown that the amoeboid forms of *E. histolytica* may survive, in cultures, for any time up to three days at ordinary room-temperatures. In order to make certain, therefore, that cultures made by inoculating cysts into fresh culture-tubes really develop from cysts alone, it is necessary to keep the cyst-inoculum for more than three days at room-temperature before making the experiment. If culture-cysts are transferred to new medium and incubated after the lapse of any briefer interval, it must always be uncertain whether the resulting amoebae are all derived from these cysts, or partly from them and partly from accidentally surviving free forms.

[1] See Dobell and Laidlaw (1926 *a*), where the composition of these media is more exactly described. [2] Strain *K.* 28 c cannot be propagated continuously in this medium.

[3] If the amoebae are transferred to plain Ringer's fluid and starch—in which they will live for many days at 37° C.—they eat the starch, but neither multiply nor encyst. If they are cultivated continuously in medium containing starch, they gradually cease to form cysts altogether. (Cf. Dobell and Laidlaw, 1926 *a.*)

[4] See Dobell and Laidlaw (1926 *a*). The culture-tubes should be incubated in the ordinary upright position—not aslant. Incubation in the fashion recommended by Yorke and Adams (1926, p. 282) has always given me inferior results with all strains which I have yet tested.

[5] See Dobell (1927).

Culture-cysts can, however, be readily freed from concomitant amoebae by treating the mixture with $N/20$ hydrochloric acid[1] for 30 minutes at room-temperature, or for 75 minutes at 37° C.[2]. Treatment for shorter times cannot be trusted to kill off all free amoebae. Cysts so treated must, of course, be inoculated into medium previously sown with suitable bacteria.

Since treatment with acid is more laborious, and kills more cysts, I usually adopt the first method. To observe excystation, and to study the subsequent stages, I have generally kept the cysts—either in the culture-tube in which they were formed, or in sterilized distilled water or Ringer's fluid—for about a week at room-temperature before inoculating them into fresh medium. Most mature cysts live thus for five to ten days, though many will live for a fortnight and some for as long as three or four weeks; while an occasional cyst may even survive for 37 days[3]. As the object aimed at is to obtain a maximum hatch, without varying the bacterial environment and with complete assurance that none of the resultant amoebae can have originated from surviving free forms in the inoculum, I have usually made my observations on untreated culture-cysts previously kept at room-temperature for six to eight days. To obtain a large quantity of cysts for experimentation I have frequently concentrated them—by sedimentation in sterile water or Ringer's fluid—and then pooled the concentrates from several cultures.

Experience has taught me that *E. histolytica* is very sensitive to changes of temperature. The amoebae live, in their natural hosts, at about 37°–38° C.; and their development in cultures is regular and normal only when this temperature is maintained. Cooling or overheating is very apt to produce abnormalities. It is therefore most important when studying critical stages in the life-history —such as division, encystation, or excystation—to keep the temperature constant. Even a slight and transient change—such as may occur in removing a culture from the incubator, and transferring its contents to a warm stage for observation—may modify, or possibly inhibit, further development. For this reason I do not rely upon ordinary warm-stage methods, but I make all my critical experiments and observations in the hot-room at the National Institute, where a temperature of 37° C. is constantly maintained. My cultures are incubated in this room, and everything used—microscope, slides, and all other apparatus and glassware—is previously adjusted to the same temperature. This method I have found entirely satisfactory so far as the amoebae are concerned, though somewhat trying to the experimenter when practised many hours a day over a long period. It is the only method, however, by which variations of temperature, at every stage, can be absolutely excluded. All the results here recorded have been obtained in this manner.

As I am now convinced of the paramount importance of the several factors just noted, I will recapitulate them here in a single sentence for the sake of emphasis. *To obtain constant and predictable results it is essential* (1) *to work with*

[1] See Dobell and Laidlaw (1926 *a*); Dobell (1927).

[2] See Dobell (1927). [3] See Dobell (1927).

a pure strain of amoebae; (2) *to study properly controlled stages only* (i.e., *amoebae or cysts—not unknown mixtures*); (3) *to make certain that the accompanying bacterial flora is invariable*; (4) *to employ a suitable and standardized medium for all cultures*; (5) *to maintain an unvarying temperature of* 37°–38° C. *throughout.* Results obtained by any less rigorous technique, and without attention to the precautions indicated, have—in my experience—little or no scientific value[1].

Cytological Technique.—The ordinary methods of fixing and staining amoebae are too well known to need redescription here. Any trustworthy cytological technique suffices for the study of *E. histolytica* in cultures: its success depends chiefly upon the skill of the technician[2]. I have tried many methods, and used none that is essentially new. In the description of the Plates the reader will find particulars of some of the fixatives and stains that I have employed, but the following supplementary notes may prove helpful.

Schaudinn's fluid (with or without the addition of acetic acid) should be used with caution for fixing cysts incubated to study excystation. It is very apt—especially when used hot—to burst them and produce artifacts. Consequently, if one wishes to study *E. histolytica* rather than "*Councilmania*" it should not be generally employed[3]. Much better fixation of the hatching stages can usually be obtained with Bouin's fluid, which seldom causes bursting or other distortions when properly used. Excellent fixation of amoebae and cysts at all stages is also obtainable with the sublimate-formol mixture of Carnoy and Lebrun (saturated aqueous solution of mercuric chloride, three parts; strong formol (40 per cent. formaldehyde), one part). I have always obtained the best results by fixing (at 37° C.) for one to two hours. Films should be well hardened in alcohol (preferably for two or three weeks at least) before they are stained and mounted, because rapid methods invariably cause shrinkage and other artifacts.

Any of the usual stains can be made to yield serviceable preparations. After trying a large number, I have found Heidenhain's iron-haematoxylin and my own alcoholic modification of this stain both very useful: but neither of these methods has given me such good results, when studying the stages following excystation, as iron-chlorocarmine (Hollande, 1916). These stages are extremely difficult to stain satisfactorily, for they consist of large and small and intermediate-sized amoebae with variable nuclear contents. Stained by ordinary iron-haematoxylin methods, films of such amoebae seldom or never give uniform results. Usually the larger individuals are black when the smaller are well stained; or, when the largest amoebae are well differentiated, the smallest are completely decolorized. But with iron-chlorocarmine, which allows much

[1] I can support this statement with extensive experimental evidence: but it seems to me unnecessary, as I am confident that every other worker who devotes serious attention to the matter must arrive at identical conclusions.

[2] I may remark here—since I observe a tendency among the rising generation of protozoologists to entrust the preparation of their material to more or less trained assistants—that I am, and always have been, my own technician.

[3] Cf. Toynbee Wight and Prince (1927), with whose conclusions I am in general agreement.

greater latitude in differentiation, it is fairly easy to obtain preparations in which amoebae of all sizes are well stained—or, at least, sufficiently well stained to allow of detailed study of them all. I have found this method most useful, and can strongly recommend it.

When cysts of *E. histolytica* are mounted in Canada balsam, their walls usually become invisible[1]. Consequently, permanent preparations of excysting stages are troublesome to investigate in this medium; for—as the protoplasm alone is visible—the appearances do not correspond with reality, as seen in the living state. I have surmounted this difficulty by using stains (such as my alcoholic iron-haematein) which tinge the cyst wall, or by mounting otherwise stained preparations in media having a refractive index different from that of balsam[2].

The optical apparatus which I have used is noted in the description of the Plates, and is of the best. All my figures have been carefully drawn with the *camera lucida*; and they are not diagrammatic—though I have, of course, purposely selected particularly suitable specimens for illustration. Most of the organisms which I am about to describe are comparatively easy to study under the microscope, but exceedingly difficult to draw correctly.

III. DESCRIPTIVE.

In this section I shall describe my observations on the development of *E. histolytica* in cultures. My description is based upon Strain *K*. 28 c, cultivated by the methods indicated, and all my figures depict this particular organism. But I must again record here expressly—to avoid any misunderstanding—that although I have intensively studied this strain alone, at all stages of development, I have now also studied scores of other strains of *E. histolytica* (simian and human, but chiefly simian) in the same way, though not in such detail or so repeatedly: and my partial observations on all these other strains—however obtained and however cultivated—agree with my more thorough observations on Strain *K*. 28 c. While I regard it as possible, therefore, that yet other strains may develop differently, my own experience leads me to believe that this is unlikely.

(*A*) THE LIFE-CYCLE AS A WHOLE.

The life-cycle of *E. histolytica* in cultures is, in general outline, quite simple. Tissue-invading phases—such as may occur occasionally in a natural environment—are necessarily absent, for the amoebae live entirely at the expense of the culture-medium and its bacterial contents. Under suitable conditions they grow and multiply and then encyst; and their cysts then liberate new amoebae, which, after a further period of division and differentiation, grow and multiply

[1] Cf. Dobell and Jepps (1918).

[2] I have devised and used numerous substitutes for Canada balsam; for it is, in many respects, a most undesirable mounting-medium. My researches in this connexion—made mainly during the last 11 years with Dr P. P. Laidlaw—will be published elsewhere. The subject is far too complex to consider here.

and encyst afresh. Nothing else happens, but the details of this simple cycle are somewhat complex.

As it is necessary to use words in describing the various stages of development, I shall first note several terms which I propose to employ—since they differ from those of some other workers.

(1) The ordinary free forms of *E. histolytica* are generally called "vegetative" amoebae; but as this adjective appears to me inappropriate[1] I shall not use it. I shall instead refer to the actively feeding and multiplying forms, occurring in the bowel or in the culture-tube, as *trophic* amoebae—to distinguish them from the encysted organisms and from those which form or which issue from cysts.

(2) I shall continue to call the organisms which form cysts *precystic* amoebae, as this term is now current. It is not entirely unobjectionable[2], but is obviously preferable to the awkward expression "preëncysted entamoebae" originally used by Walker (1913) to designate these stages.

(3) By *encystation* I mean "the act of encysting," and by *encystment* "the state of being encysted." I believe this to be conformable with the best usage, and I deplore the frequent confusion of these words[3] in recent papers.

(4) By *excystation* I mean "the act of escaping from the cyst"—or *hatching*, as it may be called more simply[4].

(5) The amoebae which hatch out of the cysts are not themselves ordinary trophic forms, but specialized individuals which give rise to trophic amoebae by undergoing a series of divisions. As these organisms are peculiar, and as I shall have to describe their development in detail, it is convenient to have a name for them. I propose to call them all *metacystic* amoebae[5]; and accordingly I shall designate as *metacystic stages* all those stages in the life-cycle which intervene between excystation and the reappearance of the trophic forms.

The complete life-cycle of *E. histolytica* in cultures consists, therefore, of the following successive stages: (1) *trophic amoebae*, (2) *precystic amoebae*, (3) *cysts*, (4) *metacystic amoebae*, which develop into trophic amoebae (1) once more. This cycle can be induced to recur *ad infinitum*—or, at all events, *ad libitum*. I shall describe it in this order.

[1] In common English, "to vegetate" means "to grow like a plant," or (figuratively) "to lead an idle or inactive life." It is therefore difficult to understand why the epithet "vegetative" has been bestowed upon the free amoebae rather than upon the encysted forms, to which it is obviously more applicable.

[2] I should prefer to use the term *procystic*; but the hybrid has, I fear, come to stay.

[3] It is, to me, difficult to understand how anybody familiar with the English language can regard them as synonymous. Surely nobody fails to realize the distinction between other exactly comparable words (*e.g.*, "excitement" and "excitation") which are in daily use?

[4] I regard the verbs "to excyst" and "to hatch" as synonymous when applied to the development of an amoeba.

[5] It is necessary to discriminate further among these amoebae, but the terminology which I shall use for this purpose will be explained in its proper place (p. 388 *infra*).

(*B*) THE TROPHIC AMOEBAE.

The ordinary amoebae of *E. histolytica* are now too well known to need redescription[1], but several points regarding the cultural forms must be noted. To avoid lengthy description I have illustrated the essentials in Plate XXII.

In their natural host—in the case of Strain *K.* 28 c a monkey (*M. sinicus*)—the amoebae are of medium size, and ingest bacteria (Pl. XXII, figs. 1, 2). They neither invade nor feed upon the tissue of the large intestine. When they establish themselves in a kitten, however, they cease to eat bacteria, and attack the lining of the gut. They then attain a larger size, and their cytoplasm becomes very clear (fig. 3). The only inclusions seen are occasional red blood-corpuscles (fig. 4), but even these are seldom ingested. When the amoebae are recovered in cultures from an infected kitten, they revert to their original food-habits[2]. In Boeck-Drbohlav cultures they ingest bacteria, and resume their original size and appearance (fig. 5): but when cultivated in media containing starch they eat both bacteria and starch, and usually attain a larger size (figs. 6, 7). Many individuals become so stuffed with starch-grains as to be unsuitable for cytological study—their nuclei often being distorted by pressure of the ingested grains (fig. 7).

Whether studied in their original host, or in an experimentally infected kitten, or in cultures made from either animal, the amoebae show the same nuclear structure. Every nucleus has a small central karyosome, and a peripheral layer of fine chromatin beads (cf. figs. 1, 3, 5, 6). But the constitution of the karyosome is difficult to determine. With some methods of fixation and staining it appears as a small spherical mass of "chromatin" surrounded by a clear zone (fig. 5), with others it commonly shows greater detail (fig. 6). The central mass of "chromatin" does not appear homogeneous, but has a tiny dot ("centriole") at its centre and several definite granules[3] at its periphery. (The "clear zone" lies externally to all these structures.) This appearance is often easily observable, but I am still in doubt as to how it should be interpreted. The area between the central karyosomic structures and the peripheral chromatin layer is usually free from chromatin; but with certain staining methods it may show a variable number of somewhat indefinite granules. In fixed specimens it has an alveolate structure, and usually exhibits a few fairly definite lines radiating from the karyosome. Both alveoli and radii are probably fixation-artifacts[4].

Figs. 1–7 (Pl. XXII) show typical trophic amoebae of Strain *K.* 28 c in the faeces of their original host (*M. sinicus*), in the dysenteric discharges of the experimentally infected kitten (No. 28), and in cultures derived from this

[1] Cf. Dobell (1919), where a detailed account is given.

[2] All the foregoing statements apply not only to Strain *K.* 28 c but to all simian *E. histolytica* which I have yet studied.

[3] These granules—of which about five are usually seen in optical section (as in fig. 6)—appear to be six to eight in number. But they are excessively minute, and very difficult to count.

[4] I mention this again because some writers seem to attach undue importance to this feature.

kitten. They were all drawn from preparations of the same strain for the sake of comparison with other stages about to be described, but they might equally well be used to illustrate any of the other strains which I have studied in monkeys, kittens, and cultures.

In its original simian host Strain *K.* 28 c, like all others, formed typical 4-nucleate cysts[1]. In the kitten no cysts were ever found—in agreement with previous experience with human and simian strains of *E. histolytica.*

Multiplication.—Like all other strains studied (both human and simian), *K.* 28 c multiplies in cultures by equal binary fission; and in no other way. I have already described this process[2]—in a human strain experimentally introduced into kittens—and can add little to that description. The chief stages are shown in figs. 8–14 (Pl. XXII)[3].

I figure only one prophase (fig. 8), two spindles (figs. 9, 10), and four telophases (figs. 11–14). This is not because I have not seen and studied very many more; but because I am still in great doubt regarding the interpretation of the earlier phases, and now wish to direct particular attention to the later stages (telophases), since their recognition is essential for understanding the metacystic development.

Although I have studied a large number of early stages of nuclear division —not only in *K.* 28 c, but in many other strains also—I am still in doubt about the origin of the chromosomes and their exact number. As I have already pointed out (1919, p. 41) the prophases and spindles are very puzzling. By selecting certain preparations arbitrarily, a more or less consistent series of stages can be made: but different selections can be combined into different series, and it is extremely difficult to ascertain the true sequence. Some of the definite granules present in prophases (fig. 8), in spindles (figs. 9, 10), and in telophases (figs. 11–13), are probably chromosomes; but in addition to these there are other granular structures whose interpretation is problematic. At present I believe that the division is a mitosis of a peculiar kind, and that the chromosome number is probably constant: but what it really is I do not know. In different specimens I have counted, quite clearly, all numbers from five to eight. I am inclined to think that the latter figure is probably the real number[4]. As regards the origin of the chromosomes, I am inclined to believe that they arise mainly from the darkly staining granules at the periphery of the "chromatic" part of the karyosome. Appearances such as are shown in figs. 6 and 8 —which are not uncommon—strongly suggest this; and the fact that eight granules are often countable at these stages supports the view that the "granules" are chromosomes and that this is their number. But I am not

[1] These are not illustrated, because they are exactly like any other cysts of the same species.

[2] See Dobell (1919), p. 40.

[3] The individuals illustrated were purposely selected from a culture containing only a little rice-starch, so that many had ingested only a few grains. Richer cultures, and more plentiful divisions, can be obtained when starch is supplied in abundance: but such amoebae, gorged with starch, are unsuitable for cytological study.

[4] According to Kofoid and Swezy (1925) the chromosome number is six.

confident about this, and must therefore leave this important point in doubt[1].

The characteristic features of the telophases are shown in figs. 12–14. They are chiefly (1) the persistent remains of the spindles (so that separating daughter-nuclei often resemble a pair of tadpoles, sometimes with their tails joined as in fig. 12): (2) the indistinctness or absence, at first, of a definite karyosome, but the presence of various scattered and stainable granules (chromosomes?): and later (3) the gradual differentiation of an eccentric karyosome (figs. 13, 14). Recently divided daughter-amoebae therefore usually show a karyosome which is not central, and a variable number of rather indefinite granular structures in the area between it and the peripheral beads (fig. 15).

No details of nuclear division can be made out in the living organism. Individuals about to divide can easily be recognized by their large size and rounded form. They remain almost motionless during division, and only begin to put out pseudopodia again when separation is taking place (fig. 14). The time occupied in the whole process—from the moment when the organism rounds up, until the daughter-amoebae finally separate—is usually about 10 to 15 minutes[2].

(*C*) THE PRECYSTIC AMOEBAE.

Precystic amoebae (Pl. XXII, fig. 16) are usually smaller than trophic forms, their size being proportionate to that of the cysts into which they are transformed. Their cytoplasm is, or becomes, free from all food-inclusions, but usually contains diffuse glycogen (easily demonstrable by its iodine-reaction) and occasionally chromatoid bodies. The structure of their nuclei is characteristic, and has already been noted[3]. Its chief peculiarities are the more or less eccentric karyosome, and the occasional presence of "chromatin" granules in the area between it and the nuclear membrane.

The precystic amoebae seen in cultures differ in no way from those which are formed in the bowel. From a study of a large number at all stages of development, I feel satisfied that their distinctive features—just noted—have a very simple explanation. The presence of glycogen and chromatoids merely denotes preparation—by storage of a reserve food-supply—for life in an inactive and encysted state: while the reduction in bodily diameter, and the telophasic characteristics of the nucleus, indicate that the precystic amoeba is the product of a recent division.

[1] I am continuing my study of the nuclear division, and hope to be able to describe it more accurately later. I am aware that some other writers (*e.g.* Kofoid and Swezy, 1925; Uribe, 1926) are more satisfied with their own work, though I do not share their confidence in their own conclusions. Kofoid and Swezy are especially critical of my earlier observations; but I am content to let others judge whether my condemned figures (1919, Pl. III) or theirs (1925, Pl. 32) show the more accurate and complete series of division-stages of the trophic forms of *E. histolytica*.

[2] This applies to all strains which I have studied—not merely to *K.* 28 c—but is only true of amoebae kept under the most favourable conditions at 37° C. continuously. Lowering the temperature at any stage may delay division indefinitely.

[3] Cf. Dobell (1919), p. 45.

(*D*) Encystation.

E. histolytica encysts in cultures exactly as it does in the intestine. As the process is now too well known to need description[1], I have given no figures of stages between the precystic amoeba (fig. 16) and the fully formed uninucleate cyst (fig. 17).

The time occupied in passing from the active precystic stage to the stage of complete encystment is difficult to determine accurately: for the cyst wall, even when fully formed, is extremely thin, so that at its first appearance it is almost invisible, while afterwards one cannot be certain when it attains its full development. From such observations as I have made, I judge that the time-interval between the stages figured (figs. 16 and 17) is probably about two hours: but I can make no exact estimate.

I have made many attempts to discover the origin of supernucleate cysts (*vide infra*, p. 382), in order to find out whether they arise from peculiar precystic amoebae; but I have hitherto failed to satisfy myself in this matter.

(*E*) The Cysts.

Culture-cysts of *E. histolytica* differ in no essential structural particular from those formed naturally in the bowel. They develop regularly from the uninucleate to the quadrinucleate (mature) state by two successive nuclear divisions. As these stages are now well known, and nobody nowadays disputes their sequence and interpretation, I shall merely illustrate a few of them without comment. (See Pl. XXII, figs. 17–21.) Everything that I have previously written[2] about the development of the cyst in the intestine I have been able to confirm repeatedly in cultures.

It has been noted elsewhere[3] that culture-cysts of *E. histolytica* (all strains) generally contain more abundant glycogen than those passed in the faeces. This is clearly because the amoebae in my cultures are more liberally fed with starch (from which they manufacture the glycogen) than those in the gut usually are. The chromatoid bodies in culture-cysts display very variable forms—just as they do in cysts formed in their natural environment.

During the last 20 months I have obtained typical cysts of Strain *K*. 28 c—like those figured—in thousands, and at many different cultural generations. Their average diameter is always approximately 13·5 μ (ranging from *ca.* 10 μ to *ca.* 16 μ), and careful measurements of a sufficient number from any culture have invariably given a unimodal curve of the same character[4].

A notable feature of the cysts formed in cultures is their uniform roundness. Oval or irregular specimens are very rare. This is doubtless because the cysts, at the time of their formation, are not subjected to unequal pressure by their

[1] Cf. Dobell (1919), p. 45.

[2] Dobell (1919), p. 45 *et seq.*

[3] Dobell and Laidlaw (1926 *a*).

[4] No curves or more detailed measurements are added here, because I intend to publish these later in another connexion.

surroundings—as those formed in solidifying faeces must often be—and are consequently able to assume and retain their natural spherical shape.

The time occupied in developing from the uninucleate to the quadrinucleate state is approximately six hours in cultures. In sealed preparations examined continuously under the microscope, development does not always proceed normally: it is often retarded, and sometimes even arrested. It is not easy to determine the time, therefore, by directly observing individual cysts. Moreover, all uninucleate cysts are not of the same age, as this stage persists for some time before the first nuclear division takes place. The time just given is inferred from repeated observations made on large numbers of cysts in cultures[1] (Strain *K*. 28 c and many others). In a normally developing culture, if most of the cysts, at a certain moment, are found to be uninucleate; then, after further incubation for about six hours, the majority will usually be found to be quadrinucleate. But as all amoebae do not encyst simultaneously, it is never possible in such an experiment to find 100 per cent. uninucleate at the beginning and 100 per cent. quadrinucleate at the end. My estimate cannot, however, be very far from the truth.

Cysts of *E. histolytica*, whether formed in the gut or in a culture, probably have a definite term of life under defined conditions. Ripe cysts normally contain variable amounts of reserve food-material (glycogen and chromatoids): when they are kept, both glycogen and chromatoids disappear—quickly at body-temperature and more slowly at lower temperatures[2]. I infer that those cysts live longest which possess most abundant food-reserves, and which are kept at a temperature so low that the rate of metabolism is minimal.

Cysts formed in cultures do not all behave alike when incubated continuously. Those formed early—while the medium is still suitable for further growth—may hatch in the same culture. Those formed late—when the medium is exhausted—usually die unless they are transferred to a fresh culture-tube and again incubated. In any liquid which is not injurious—such as culture-medium, distilled or tap-water, or Ringer's fluid—mature cysts can live at ordinary room-temperatures for two or three weeks, and exceptionally for four or even five. At 37° C., however, they can survive in water for only a few days at most. Drying kills them instantly[3].

The glycogen and chromatoids have usually disappeared (*i.e.*, the food-supply has been used up) in most cysts kept at room-temperatures for more than a fortnight. After that time life for the majority becomes precarious, and only a small and rapidly decreasing proportion is able to survive. No nuclear changes occur in ripe living cysts during their extra-corporeal or extra-cultural existence at room-temperatures.

[1] I have made similar observations many times, also, on cysts derived from faeces.

[2] I noted this long ago, and have since confirmed the observation repeatedly. As other workers have since made similar statements—usually without reference to mine—I presume that they have repeated my experiments and obtained concordant results.

[3] Cf. Dobell (1927), where experimental evidence for the statements made in this paragraph is adduced.

If immature cysts are cooled to room-temperature they cease to develop: and if they are kept at this temperature for more than a short time, they are unable to continue their development on reincubation. Consequently, in experiments made in the way previously described[1]—in which culture-cysts are kept at room-temperature for about a week before hatching them—only the 4-nucleate cysts continue to develop and ultimately hatch. All the 1-nucleates and 2-nucleates degenerate and die.

Supernucleate Cysts.—When the 4-nucleate stage is attained, the cysts of *E. histolytica*—both in the test-tube and in the bowel—do not usually develop further. But occasionally one or more of the nuclei may divide again, and thus give rise to a cyst containing more than the normal number.

The commonest variation of this sort is seen when all four nuclei divide once more, thus producing an octonucleate cyst (fig. 22). Such cysts are formed occasionally by every encysting strain of *E. histolytica* that I have yet studied in culture. They are usually larger (15–18 μ) than the normal 4-nucleate cysts, but otherwise closely similar. I find them constantly in encysting cultures of Strain *K*. 28 c, about 1 per cent. of all cysts being thus constituted. In different cultures, however, the percentage of 8-nucleates ranges from about 0·5 to 3·0.

Similar octonucleate cysts of *E. histolytica* also occur at times in the faeces of man[2] and monkeys (*M. sinicus* and *M. rhesus*); and they are probably commoner than is usually supposed, as they can easily be mistaken for cysts of *E. coli*. I have found them not only in cultures[3] of Strain *K*. 28 c, but also—though very seldom—in the faeces of the monkey from which this strain was derived.

Supernucleate cysts containing six or five nuclei also occur very rarely in cultures of *E. histolytica*. The 6-nucleate cysts (Pl. XXV, fig. 95) originate from ordinary 4-nucleates in which two of the nuclei have undergone a further division; and they always show, accordingly, two large nuclei—of normal size—and four small (daughter) nuclei. The 5-nucleate cysts, which are much rarer, contain three large nuclei and two small, and arise from a 4-nucleate cyst in which one nucleus has divided into two. Neither in size nor in any other character do such cysts otherwise differ from normal 4-nucleates. I have found both kinds in faeces as well as in cultures, though in faeces they are excessively rare.

[1] See p. 373 *supra*.

[2] I stated this clearly in 1919; cf. p. 51 "I think it certain, therefore, that *E. histolytica* sometimes forms supernucleate cysts containing 8 nuclei." Knowles (1928, p. 73) unintentionally misrepresents me when he says that I denied having seen such cysts. The statement of mine to which he evidently refers was made about *E. ranarum*—not *E. histolytica*.

[3] It might possibly be suggested that the 8-nucleate cysts in my cultures are really cysts of *E. coli*, with which species Strain *K*. 28 c is (supposedly) contaminated. But the history of this strain, the morphology of the cysts themselves, and the occurrence of supernucleate cysts of intermediate types, conclusively negative such an interpretation.

Sexnucleate cysts occur so seldom in cultures of Strain *K*. 28 c that it is difficult to determine their frequency. Perhaps one cyst in a thousand may attain this condition: but this is only a rough estimate. Quinquenucleate cysts are so rare that I cannot make even a plausible guess at their frequency. I have not seen more than half a dozen among hundreds of thousands of cysts that I have examined from stools and cultures.

A cyst containing seven nuclei—one large and six small, resulting from the division of three of the four nuclei in a normal cyst—I have only once seen. It is so rare as to be negligible. I have never found a cyst containing more than eight nuclei.

Both 8-nucleate and 6-nucleate cysts are viable and able to hatch. Regarding the 5-nucleates I have no evidence; but it seems probable, on analogy, that they also are capable of further development.

(*F*) EXCYSTATION.

As I have now been able to observe the excystation of *E. histolytica* very frequently in cultures, and have studied all stages in many hundreds of cysts—both alive and in good permanent preparations—I can describe the process with some confidence.

My description and figures here, as before, are of Strain *K*. 28 c: but I have studied cysts of many other strains, from faeces and from cultures, in the same way, and I attach particular importance in this connexion to my confirmatory observations on Strains *DKB*. and *DMK*.[1]. As all three strains excyst in the same way under similar conditions, I am persuaded that the process which I am about to describe is not abnormal.

During excystation the entire protoplasmic contents escapes, as a single quadrinucleate amoeba, through a minute perforation in the wall of the cyst. I shall now describe this process in detail.

Mature living cysts, when incubated in a suitable medium, do not all hatch simultaneously. Some hatch quickly, others slowly. Moreover, all strains do not develop at the same rate. The times which I shall give are those characteristic of Strain *K*. 28 c, cultivated in the way described, and they are the average times observed in many different experiments. It must also be noted that the cysts, in all these experiments, were inoculated into medium not previously warmed. They were always kept at room-temperature for about a week[2], then inoculated into culture-tubes at the same temperature, and then incubated at 37° C. Consequently, when I speak of a culture as incubated for (say) three or four hours, I mean one made in this manner. A little time is needed to raise the original temperature to that of the body, and allowance must therefore be made for this initial period of warming[3].

[1] See p. 370 *supra*.

[2] For reasons already explained. See p. 373 *supra*.

[3] Cysts inoculated into warm medium (37° C.) develop equally well, but I have not studied their rate of development in equal detail.

During the first two hours of incubation the chromatoid bodies[1] gradually become smaller and stain less intensely, and usually appear granulate or fibrillate instead of homogeneous. (Cf. Pl. XXII, figs. 23, 24.) Finally they disintegrate and disappear completely, and the protoplasm thus becomes very clear. Cysts do not hatch until their chromatoids and glycogen have been absorbed.

Soon after the chromatoids have vanished, the protoplasm begins to show streaming movements. It then gradually separates from the cyst wall, until the whole organism lies suspended freely in its envelope (fig. 25). At this stage it becomes very active—moving about inside its cyst as though impatient at being confined. Clear ectoplasmic pseudopodia are formed at various points, and travel rapidly round the periphery. Frequently they press against the wall at certain spots, as though the imprisoned organism were searching for some exit. Sooner or later the movements become slower, and a pseudopodium is then seen to be applied to one point on the internal surface of the cyst wall. Shortly afterwards its tip squeezes through a minute and previously invisible pore at this spot, and appears like a tiny hernia on the outside (figs. 26, 27). I do not know how the perforation is made, but imagine that the amoeba secretes a ferment which dissolves the cyst wall. The protoplasm now rapidly exudes through the pore (figs. 27–30), but the whole organism never emerges immediately in this manner. When a small portion has flowed out, it begins to flow back again; and the whole of the protoplasm re-enters the cyst once more —only a minute bead (as in fig. 27) remaining outside. This operation is then repeated many times; but on each occasion more protoplasm emerges (figs. 30–32) and less returns, until finally the whole organism escapes from its envelope, though the tip of its "tail" generally remains caught in the pore (fig. 33). When this stage is reached, the amoeba does not return. It begins to creep off, dragging the empty cyst behind it.

The nuclei pass through the pore one at a time; but it is so narrow that they are greatly distorted in the process (fig. 29), though they soon recover their shape and appear to be unharmed by the violent constriction which they suffer (figs. 29–31). When the nuclei have emerged, they run together as though magnetically attracted, and remain closely clumped during the ensuing stages (figs. 32–35).

As already implied, the pore through which the organism escapes is so extremely minute as to be almost invisible—either in living specimens or in well fixed and stained preparations: but in the latter it sometimes appears to have a slightly thickened and more stainable rim (fig. 29). In life the fine isthmus of protoplasm connecting the exocystic and endocystic portions of the organism is usually difficult to make out, though one can easily see either portion increase in volume as the other diminishes.

[1] Cysts kept at room-temperature for a week have usually used up all their glycogen; but if any is still present, it disappears rapidly on incubation. Culture-cysts sometimes become more or less vacuolate on keeping; but even those full of vacuoles often hatch—their vacuoles gradually disappearing during excystation.

Excysting amoebae are evidently very hungry, for they begin to ingest food before they have hatched completely. (Cf. fig. 32, which shows an incompletely excysted organism that has already taken up two starch-grains and several bacteria.) Neither the ingested food-bodies nor the escaped nuclei enter the cyst with the periodic refluxes of the protoplasm. They always remain in the exocystic portion of the organism.

When the amoeba is moving about inside, or flowing in or out through the pore, the cyst wall is frequently deformed—becoming oval or irregular in contour, as a result of the varying pressures exerted on it by the active organism in its struggle to escape. But it remains relatively rigid, and never tears, bursts, collapses, or becomes indented.

The space between the protoplasm and the wall of the cyst is at first small (figs. 25–30), but later becomes larger (figs. 31, 32). It is filled with liquid, and contains a few very minute granules which are left in the cyst after the escape of the amoeba. These granules can be readily stained during life with neutral red, and are present at all stages. I imagine them to be an excretion of some sort. (A few are visible in fig. 32.) In fixed specimens, the empty or emptying cyst also sometimes shows faint strands of coagulated matter (fig. 32). These are probably artifacts produced by precipitation of minute amounts of protein in the endocystic liquid, which in living cysts appears perfectly hyaline.

The excysted amoeba (fig. 33), with the tip of its "tail" still nipped in the pore, generally creeps about for some time, trailing the empty cyst behind it, before it gets free of its encumbrance. It does so finally, however, and then moves off with great rapidity. Frequently a minute bead of exuvial matter (? protoplasm) is left inside the pore when complete severance occurs: but occasionally a lump of cytoplasm—sometimes as big as the portion inside the cyst shown in fig. 32—is sacrificed by the organism in making its final escape. Very rarely two pores are formed by an excysting amoeba, and the protoplasm is then extruded through both. But after a time it flows in completely through one, and excystation is effected through the other.

When excystation is complete, the discarded cyst wall remains as a perfectly spherical vesicle, containing nothing but hyaline liquid and a few excretory granules and the tiny bead, or bit of protoplasm, detached from the "tail" of the departed amoeba. The pore through which escape was effected is usually invisible—even with most careful scrutiny. It closes up completely. But occasionally it can be seen clearly, as a very minute aperture with slightly introverted edges. Such empty cysts have a very characteristic appearance. They persist for some hours, and then disintegrate and vanish. Careful examination of these empty cysts has always failed to convince me that the cyst wall is composed of more than one layer.

The time occupied in excystation varies. From the moment when the organism first begins to move in its cyst (fig. 25) until it emerges completely (fig. 33) about half an hour commonly elapses. But I have seen cysts hatch in as little as 15 minutes, and others which took more than an hour.

Some cysts show the first signs of hatching after incubation for only 2¼ hours; and from the third to the fifth hour numerous hatching cysts, in all stages, can usually be found. After this time—up to about the tenth hour of incubation—only isolated individuals excyst; and those cysts which have not hatched by the twelfth hour usually perish. Nevertheless, successful excystation may occur much later. I have already recorded one instance in which I observed it after 17½ hours[1], and I have since found—in a stained preparation—an organism which had only just hatched (a stage closely similar to fig. 33) after incubation for 20½ hours. But such late excystations are rare exceptions.

The phenomena just described surprised me when I first observed them; but after seeing them many times, and on thinking about them, they seem to me almost too obviously natural to need any explanation. The cyst wall is a very thin, but tough and elastic membrane, as is shown by its behaviour. For a soft mass of protoplasm to extricate itself from such an investment, through a minute hole, is not easy. It is, in fact, an interesting little problem in hydrostatics—a problem which *E. histolytica* (and other amoebae too) has solved very neatly. The entire cyst is comparable with a thin but uncollapsible rubber ball completely filled with a mass of soft jelly: and the problem is how to extract the jelly—*in toto* and without injury—through a pinhole made in the rubber. It is clear that if the jelly were to begin to exude through the perforation—which it entirely blocks—it would quickly be arrested by the formation of a vacuum between itself and the envelope. If it were partly pulled out by force, and then released, it would be sucked back by the negative pressure inside.

I have very little doubt that the repeated flowing in and out of the organism during excystation is a kind of pumping action whereby water is drawn in through the cyst wall—not through the pore, which is plugged by protoplasm—in order to adjust the internal pressure to that outside. That water *can* pass into the cyst through the unbroken wall is certain: for if intact cysts are placed in a weak solution of neutral red their excretory granules (and sometimes their protoplasm) become stained[2]. Since the stain diffuses through in solution, it is clear that water must also pass into the cyst.

During the early stages of incubation, prior to excystation, the cyst probably swells slightly as a whole. This is difficult to determine by accurate measurement; but cysts during these stages are often above the average size (cf. figs. 23 and 24 with figs. 17–21). When the protoplasm contracts from the wall later, however, they usually appear slightly smaller again (fig. 25). It is probable, therefore, that water first diffuses into the cyst, which then expands slightly—its wall being elastic; and the protoplasm then contracts, expelling the absorbed liquid, which thus comes to lie between the organism and its

[1] Dobell (1927), p. 296. I have also there given some cultural evidence indicating that excystation may take place after incubation for 24 hours: but I have never observed this directly.

[2] Such cysts are not injured by the dye, and hatch normally. I have hatched cysts whose contents were coloured quite a bright pink, and they produced excellent cultures later.

envelope. When the protoplasm begins to exude through the pore it is soon arrested by the negative pressure within. More water is then sucked through the cyst wall, while the protoplasm at the same time partly returns. The pressure within the cyst is no doubt regulated by the elasticity of the wall. Anyway, the appearances of cysts at these stages—sometimes slightly swollen (as in fig. 26), sometimes compact (fig. 28)—are very suggestive. When the organism has finally escaped (*e.g.* fig. 33), the empty cyst generally appears slightly smaller, owing to the resumption of its natural unstrained form and size. Its elasticity is also clearly evinced by the constriction of the pore—whereby the tip of the "tail" of the escaped amoeba is firmly nipped and held—and its obliteration when the organism finally becomes free.

Whether or no this explanation be correct, normal excystation always occurs in the way I have described. The organism always emerges as a whole: it always comes out through an extremely small perforation in the cyst wall: it never does so all at once, but gradually—flowing out and returning many times in the process: and the empty cyst is finally abandoned almost intact[1].

During excystation the four nuclei often show slight modifications of size and structure in stained specimens (see the figures). These doubtless result from the varying pressures to which they are subjected by the activity of the whole organism. The nuclei can be seen in life, and are carried about passively in the flowing protoplasm. They do not divide, fuse, extrude chromatin, or undergo any notable change: and they do not become clumped together until excystation is complete. These points are illustrated in my figures[2], which were all drawn from typical specimens.

(*G*) THE METACYSTIC AMOEBAE.

When ripe cysts of *E. histolytica* are incubated in suitable medium, they hatch—as just described—and liberate small quadrinucleate amoebae. If these are cultivated further, they ultimately produce a culture of ordinary large uninucleate trophic forms. I shall now describe how this takes place.

Hitherto it has generally been supposed that a cyst of *E. histolytica* gives rise, on hatching, either to four uninucleate amoebae or to a single quadrinucleate which somehow divides later into four uninucleates. I have just shown that the first supposition is wrong: I shall now show that the second is also. Every cyst liberates a single quadrinucleate amoeba, but this never divides into four uninucleates. The metacystic development is unlike anything that anybody has previously postulated. It is extremely difficult to study in every stage, and not easy to describe succinctly. To shorten my description I shall have to use a few new terms and some simple symbols, but these I shall first explain.

It will greatly help the reader to understand the ensuing description if

[1] Contrary statements made by other observers will be considered later (Section IV).

[2] All these figures were drawn from fixed and stained specimens, because the rapidly moving living organisms are, for me, far too difficult to draw accurately with the *camera lucida*.

he will bear the following six points—which anticipate much that will only become evident in the sequel—constantly in mind:

(1) The metacystic development is a process of subdivision only, and no conjugation or fusion of individuals or nuclei occurs.

(2) The newly hatched quadrinucleate amoeba does not divide into uninucleates directly, but by successive stages.

(3) *The original quadrinucleate amoeba does not finally give rise to four uninucleates, but to eight.* This is the key to the whole process.

(4) Every division of the cytoplasm is preceded by a nuclear division[1].

(5) Each of the four original nuclei divides once during the metacystic development—and once only.

(6) Between any two successive fissions of the cytoplasm a variable period of growth supervenes.

A recently hatched amoeba (*e.g.* Pl. XXII, fig. 35) contains four nuclei—those present originally in the cyst. I shall call these *cystic nuclei*, and designate them symbolically by the capital letter *N*. During the metacystic stages each of these nuclei divides once into two *daughter-nuclei* (see (5) above). I shall denote each of these by the small letter *n*. Every *N* nucleus is thus the parent of, or ultimately equivalent to, two *n* nuclei ($N = 2n$).

I shall call all the metacystic organisms *amoebae*, and their final products *amoebulae*: and I shall use the customary[2] adjectives of Latin derivation to express the number of nuclei present in each individual. Accordingly, I can summarize the metacystic stages as a whole (see (3) above) by saying that an originally *quadrinucleate amoeba* (having the nuclear constitution *NNNN*) ultimately produces eight *uninucleate amoebulae* (each containing one *n* nucleus).

As there are many different kinds of metacystic amoebae, containing various nuclear combinations, and as each has to be distinguished, it is convenient to have descriptive names for them. Many of these amoebae can be formed in several different ways, so that their immediate ancestry cannot always be known with certainty. But each produces a definite number of progeny (*amoebulae*), so that their final future development can always be predicted. I shall therefore name them according to their generative capacity. For this purpose I shall use distinctive substantives of Greek origin: and I shall call an amoeba which eventually produces two, three, four, five, six, seven, or eight amoebulae, a *digen*, *trigen*, *tetragen*, *pentagen*, *hexagen*, *heptagen*, or *octogen* respectively.

In illustration of what has just been said, and to show the verbal economy of such a terminology, I may again summarize the whole metacystic develop-

[1] There is one exception to this rule; but it will, I think, be easily understood from the description which follows.

[2] Customary, that is, with careful writers. Monstrosities such as "mononuclear" (for uninucleate), "tetranuclear" or even "four-nuclear" (for quadrinucleate)—and dire confusion of the adjectives *nuclear* and *nucleate* generally—are becoming increasingly fashionable, but are obviously neither good English nor good words in any language.

ment by saying that the newly hatched amoeba (Pl. XXII, fig. 35) is a *quadrinucleate octogen* (*NNNN*). This means that the *amoeba* itself contains four *cystic nuclei* (*N*), each of which will subsequently divide into two *daughter-nuclei* (*n*), which will each ultimately form the sole nuclear component of a single *uninucleate amoebula*—eight in all.

If the reader will give careful attention to the few foregoing paragraphs, he will, I hope, have little difficulty in understanding the following description.

As already noted, all cysts do not hatch simultaneously. But as the hatched amoebae continue their development independently, many different metacystic stages occur simultaneously in every culture. To sort these out, and understand their relations to one another, is far from easy. The reader can obtain some slight idea of the complexity of the problem by looking at the figures on Plates XXIII and XXIV. Any of the stages there shown—and also those in figs. 23–35 (Pl. XXII) and figs. 87–92 (Pl. XXV)—may be found in a 6-hour culture made from cysts. In the plates the organisms are arranged in some order, and links connecting different stages are put, as far as possible, in their proper places. In cultures the stages are all mixed in disorder, and the connecting links are frequently undiscoverable.

A normally developing culture of *E. histolytica* made from ripe cysts usually contains—in addition to cysts and various hatching stages—nothing but 4-nucleate amoebae during the first few hours of incubation. After incubation for a day, however, it is stocked almost entirely with abundant large uninucleate (trophic) forms. At intermediate times, amoebae with intermediate numbers of nuclei are to be found. The original quadrinucleates are replaced by trinucleates, binucleates, and uninucleates, which vary greatly in relative numbers at successive periods. But the development, in general, is fairly uniform. The longer the time of incubation, the fewer the nuclei in the majority of individuals.

To illustrate this, I give, in the accompanying Table, a series of actual counts which I made of the amoebae (Strain *K*. 28 c) present in cultures of different ages.

Table.

Duration of incubation (hours)	Total No. of amoebae counted	Percentages of amoebae			
		4-nucleate	3-nucleate	2-nucleate	1-nucleate
4	150	96·7	1·3	1·3	0·7
5	1000	69·3	8·9	13·1	8·7
6	500	65·6	11·4	12·4	10·6
7½	500	35·0	13·8	23·0	28·2
9	500	11·2	14·0	24·8	50·0
11	1000	0·6	0·3	1·9	97·2
23	500	0·4	0·0	0·2	99·4

The amoebae here enumerated were all counted in well fixed and stained permanent preparations[1]. Dividing individuals, which form a relatively very

[1] Every individual has to be carefully scrutinized, under a high magnification, in order to determine its nuclear constitution exactly; and it is not possible to ascertain details accurately in living organisms or those roughly fixed in iodine solution.

small proportion of the total population at a given moment in any culture, were excluded[1]. Percentages are calculated to the nearest first decimal place.

Figures such as these—which are most laborious to compile—merely show that the average number of nuclei present in the individuals forming the total population, at any given moment, diminishes with continued incubation. They cannot be used for any other statistical purpose, and tell us nothing about the way in which the multinucleate amoebae develop into uninucleates; because the organisms classified in this manner are not classified naturally. Amoebae containing four nuclei are not all equivalent, and consequently they cannot properly be grouped together: and the same is true of amoebae possessing one, two, or three nuclei apiece. There are actually four different kinds of quadrinucleates, three kinds of trinucleates, three kinds of binucleates, and two kinds of uninucleates. Each of these has a different ancestry and a different destiny.

The original quadrinucleate amoeba (*NNNN*) may begin its development in several different ways; and at each successive stage alternative lines of future development may be followed. Consequently, no simple, single, and invariable scheme can be made to illustrate the metacystic history as a whole. I shall therefore describe, in some detail, the commonest method of development, and shall then describe the chief variations more shortly with the aid of symbols.

Although development may follow so many different lines, it is always orderly: and the divisions—with certain easily intelligible exceptions to be noted later—are effected on a definite principle. Each cystic nucleus (*N*) divides once into two daughter-nuclei (*n* and *n*), and every cytoplasmic division is preceded by one such nuclear division. The daughter-nuclei always pass into different descendants, one going into either daughter-amoeba. Resting nuclei —whether cystic nuclei (*N*) or their daughters (*n*)—which may be present in various numbers at the time of cytoplasmic division, are distributed passively and at random to the daughter-amoebae. As will be seen presently, this accounts for the numerous different nuclear combinations found in individual organisms in the later stages.

The commonest method of metacystic development is as follows:

(1) The excysted quadrinucleate amoeba (Pl. XXII, figs. 33, 34), which commonly measures about 11 μ in diameter when rounded, creeps about actively for some time and feeds voraciously. As a result, it grows in two or three hours into a large organism measuring some 16–20 μ (fig 37, Pl. XXIII). All intermediate stages (fig. 35 (Pl. XXII) and Pl. XXIII, fig. 36) can easily be found in good cultures. The four cystic nuclei (*NNNN*) which it contains increase in size from about 2 μ to about 4 μ in diameter. They remain adherent to one another when undisturbed, though with the active movements of the organism

[1] In making these counts, amoebae with more than four nuclei were likewise excluded. The 5-nucleates occasionally seen were regarded as division-stages; and 6-nucleates and 8-nucleates, of which several were encountered, were regarded as aberrant forms and for that reason excluded also. Their origin will be described later. The inclusion of these forms would make no significant difference to the figures, and would encumber the Table with superfluous detail.

they occasionally get separated. But such separation never lasts long. They soon run together again, as though magnetized. During this period of growth they undergo no notable structural change, and remain of approximately equal size.

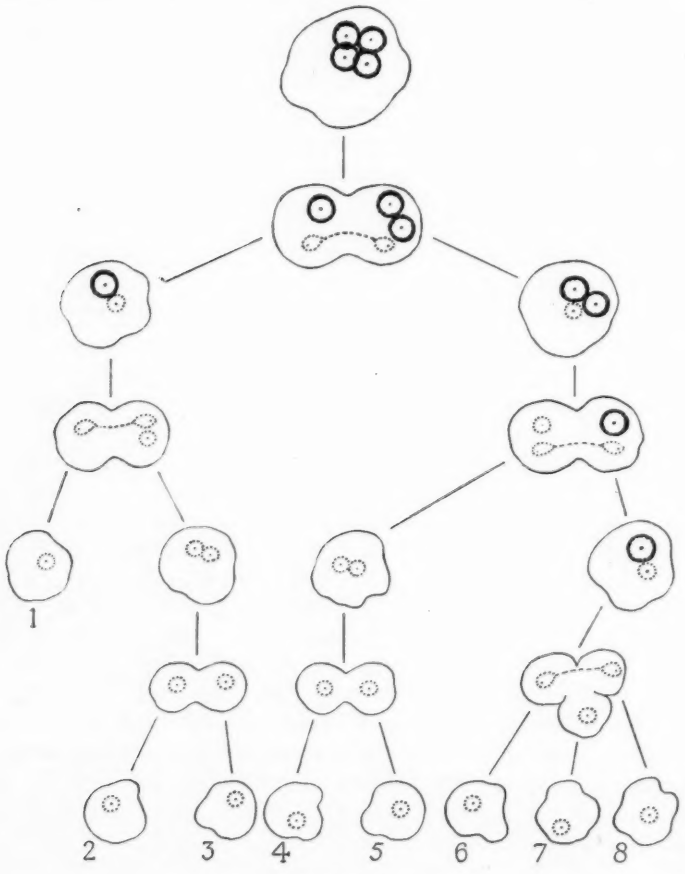
When the excysted organism has attained its full growth, one of its four nuclei begins to divide (Pl. XXIII, fig. 42)—the others remaining at rest. Division of this nucleus is then effected in the usual manner, the stages differing in no way from those seen in the trophic forms. During the later phases the whole organism becomes rounded (fig. 43), and at this time some separation of all the nuclei usually occurs. They appear temporarily to lose their attraction for one another (cf. figs. 42, 43). In the late telophases the daughter-nuclei draw apart, and the organism becomes elongate, just as it does during fission of the trophic forms (cf. figs. 43, 44 (Pl. XXIII) with (Pl. XXII) figs. 11–13). A transverse median constriction of the cytoplasm now becomes visible (fig. 44) and gradually deepens (fig. 45), until bodily fission into two is effected (fig. 46). The three non-dividing cystic nuclei are passively parcelled out to the two daughter-amoebae—one receiving one, the other two (figs. 44–46). Consequently, when bipartition is complete the daughter-individuals are not identical. One (Pl. XXIV, fig. 59) contains two cystic nuclei and one daughter-nucleus (thus having the constitution *NNn*), while the other (Pl. XXIV, fig. 72) contains one cystic nucleus and one daughter-nucleus (*Nn*). Using the terminology already explained, therefore, we can describe this first division as the bipartition of a quadrinucleate octogen (*NNNN*) into a trinucleate pentagen (*NNn*) and a binucleate trigen (*Nn*).

These two daughter-amoebae then feed and grow and later again divide on the same principle. One cystic nucleus (*N*) divides, and any other nuclei present remain at rest and are distributed to the daughter-individuals at random. In the trinucleate individual (*NNn*; Pl. XXIV, fig. 59) one large (cystic) nucleus divides (Pl. XXIV, figs. 62–64), and cytoplasmic fission follows (fig. 65). If the non-dividing nuclei are equally distributed, as they usually are, this produces (fig. 65) two binucleate amoebae—one containing a cystic nucleus and a daughter-nucleus (fig. 72), the other two daughter-nuclei (fig. 73). These are respectively, therefore, a binucleate trigen (*Nn*), and a binucleate digen (*nn*).

Individuals having the nuclear constitution *Nn* (binucleate trigens), whether formed at the first division or the second, are indistinguishable and develop in the same ways. Their cystic nucleus (*N*) divides (figs. 75, 76) like any other nucleus; cytoplasmic fission then occurs; and the original resting daughter-nucleus (*n*) is passively apportioned to one or other of the resultant individuals (figs. 77, 78). Such a division therefore produces one organism with one daughter-nucleus (fig. 84) and one with two daughter-nuclei (fig. 73). I can express this otherwise by saying that the binucleate trigen (*Nn*) divides into a binucleate digen (*nn*) and a uninucleate amoebula (*n*). It frequently happens, however, that at this division cytoplasmic tripartition—instead of bipartition—occurs (fig. 79): and the whole amoeba thus gives rise to three amoebulae (*n*, *n*, *n*) simultaneously.

Binucleate digens (*nn*, fig. 73), however formed, again feed and grow somewhat, and finally divide by simple constriction of their cytoplasm into two equal uninucleate amoebulae (figs. 82, 83).

The final result of these divisions is, as will be evident, the production of eight uninucleate amoebulae (*n*) from the originally quadrinucleate amoeba (*NNNN*) which hatches from the cyst. The whole development just described is summarized diagrammatically in Text-fig. A.



Text-fig. A.

Although the amoebae continue to feed and grow during the whole of this period, the later generations gradually become smaller—as will be obvious from the figures on Plates XXIII and XXIV. But they have no fixed size at any stage except the last. The final amoebulae are always very small, and commonly measure about 8 μ in diameter when rounded.

It has already been noted that all the nuclei, in multinucleate amoebae, separate at the moment of division. But after fission of the cytoplasm they always run together again in the daughter-individuals, so that the nuclei are characteristically aggregated at all interdivision stages (cf. figs.).

From the foregoing account it will be evident that the number of divisions which may occur between the original 4-nucleate stage (*NNNN*) and the production of an amoebula (*n*) is variable. For example, the amoebula labelled 1 in Text-fig. A is a product of two divisions; while that labelled 8 has resulted from three[1]. Again, the nuclear constitution of any particular amoeba may afford no certain clue to its ancestry. For example, it will be seen (Text-fig. A) that a binucleate trigen (*Nn*) can be formed in at least two different ways and at two different generations[2].

At all stages the daughter-nuclei can be recognized by their smaller size. During and immediately after cytoplasmic fission they are, of course, in telophase; and this affords an unmistakable means of identification. A glance at the figures will demonstrate these points. For example, fig. 59 (Pl. XXIV) is obviously an amoeba with two cystic nuclei and one daughter-nucleus (trinucleate pentagen, *NNn*): and fig. 65 clearly shows the division of a similar amoeba into a binucleate trigen (*Nn*) and a binucleate digen (*nn*). The original cystic nucleus (*N*) is here easily recognizable by its large size (fig. 65, upper individual): the two small telophasic nuclei—one in each daughter-individual —are obviously products (*n*, *n*) of the division of the other cystic nucleus (*N*): and the small resting nucleus in the lower individual is clearly a daughter-nucleus (*n*) produced at a previous division.

When all the nuclei are at rest and equal in size, it cannot always be determined whether they are cystic nuclei or daughter-nuclei: since these, owing to the growth which occurs between divisions, are only recognizable with certainty by their relative dimensions. For example, it may sometimes be doubtful whether a binucleate amoeba with two equal nuclei—neither as small as those in fig. 73 (Pl. XXIV) nor as large as those in fig. 74—is a large binucleate digen (*nn*) or a small binucleate tetragen (*NN*). But in practice it is seldom difficult to decide, and only occasional binucleate and uninucleate individuals are hard to interpret.

Cytoplasmic fission is not always equal. The products of any division are frequently of different sizes, but their dimensions are roughly proportionate to their nuclear contents. Trinucleate amoebae, for example, are generally larger than uninucleates: but daughter-amoebae containing cystic nuclei (*N*) are usually larger than those containing only daughter-nuclei (*n*). Amoebae with large nuclei (*N*), or with many nuclei, thus tend to be larger than those whose nuclei are small (*n*) or few.

The method of subdivision just described is the commonest: but—as

[1] As will be shown presently, amoebulae may also be produced by only one division, or result from as many as four.

[2] It can also be formed in at least three other ways, as will be evident a little later.

already noted—the original 4-nucleate amoeba (*NNNN*) does not always develop in the same manner. It may begin, in fact, in at least five different ways. The four other methods, in addition to the one described, are as follows—in order of frequency in my preparations:

(2) At the first division, when a cystic nucleus (*N*) divides (Pl. XXIII, figs. 42, 43), one daughter-nucleus (*n*) passes, as always, into either daughter-amoeba; but one of these then receives all three of the undivided cystic nuclei (fig. 47 shows an early stage). This results in the formation of a uninucleate amoebula (*n*) and an amoeba containing three cystic nuclei and one daughter-nucleus (quadrinucleate heptagen, *NNNn*; fig. 38).

(3) When one nucleus divides in the original amoeba (*NNNN*), the cytoplasm does not subsequently divide into two but into three daughter-individuals (Pl. XXIII, figs. 43, 54, 55). These have the same constitution in all specimens which I have yet found, and it is somewhat peculiar: namely, a uninucleate digen (*N*), and two binucleate trigens (*Nn*).

(4) Rarely the first nuclear division (figs. 42, 43) is not followed by any fission of the cytoplasm. The two daughter-nuclei run together (fig. 48), and all the nuclei then become aggregated. This produces an amoeba with five nuclei (quinquenucleate octogen, *NNNnn*; fig. 41). Such amoebae are rare in my cultures, but occur constantly[1]. They develop later in the same ways—allowing for their nuclear difference—as ordinary *NNNN* amoebae.

(5) Very rarely the first nuclear division of the *NNNN* amoeba may be followed by fission of the cytoplasm into neither two nor three but into four daughter-organisms. I have found only one such specimen (fig. 58). The products here—formed from an unusually large individual—have the nuclear constitution *NN*, *N*, *n*, and *n*.

The five alternative methods by which an excysted quadrinucleate amoeba can begin its development[2] are shown diagrammatically in Text-fig. B.

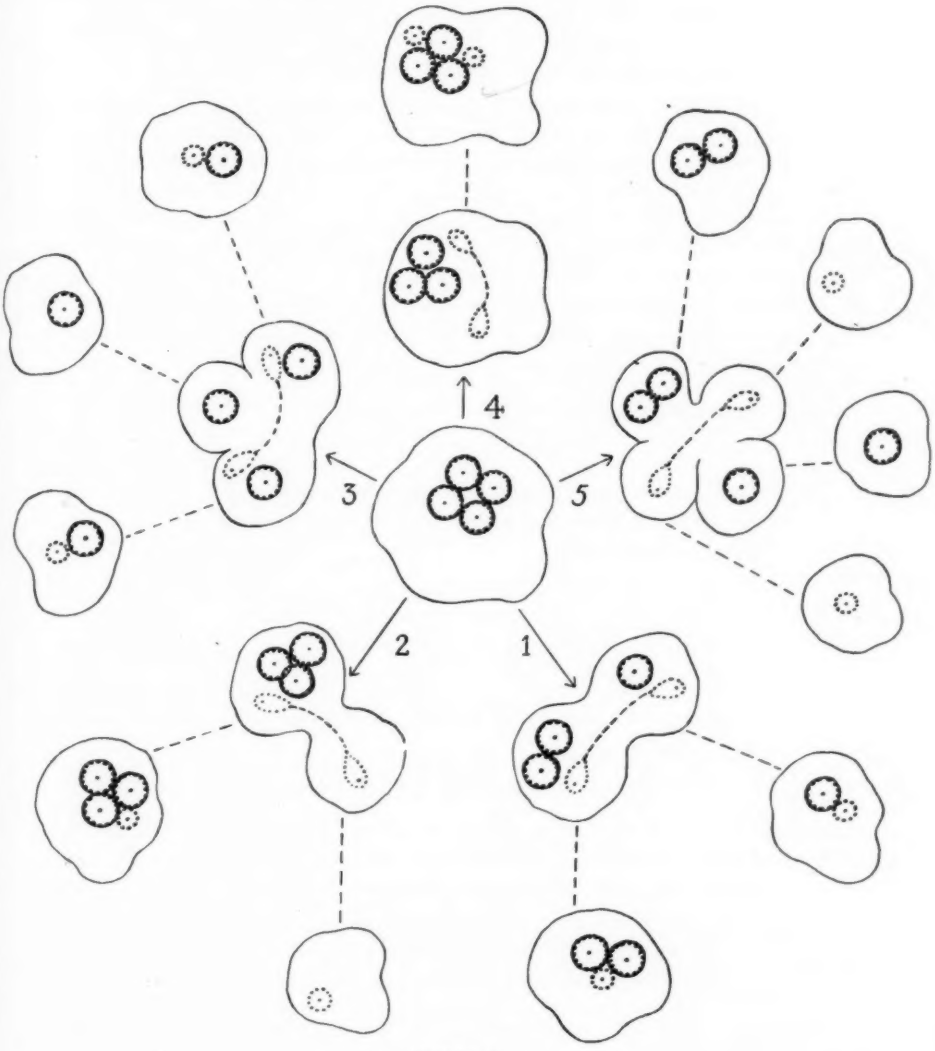
Now that I have described the metacystic development in its usual course, and have indicated the sort of variations to be expected, I shall give[3] a more

[1] It is possible that some of these 5-nucleates are derived from 5-nucleate (supernucleate) cysts, as they have the same nuclear constitution (*NNNnn*). But such cysts are excessively rare, and most 5-nucleate amoebae undoubtedly arise as described above.

[2] It may possibly be able to develop in other ways also, but these are all that I have yet discovered after long search and examination of many thousands of metacystic amoebae.

[3] I have found all the amoebae and nearly all the division-stages enumerated, and possess permanent preparations of them: while of the more usual forms of division I possess not only the main stages but a full series of intermediates. The divisions which I have not actually observed will be duly noted. Unfortunately it is not practicable to show more than a selection of the most important stages in the figures on the Plates; but I have chosen the specimens illustrated so as to show as many features as possible. Nuclear and cytoplasmic divisions take place in a similar manner at all stages, and the reader will therefore, I hope, be able to understand details which are not drawn at one stage by reference to another where they are. As the figures are all accurately drawn to scale, and show many particulars not specifically mentioned in the text, a careful study of them will help the reader to understand what follows.—In this synopsis of the metacystic development, the 5-nucleate forms already described are not included. They will be considered separately afterwards.

comprehensive account of it with the aid of the terminology and notation already explained. This account is—to the best of my knowledge—complete, and includes all the stages already described.



Text-fig. B.

It will be evident that amoebae containing various numbers and combinations of cystic (*N*) and daughter (*n*) nuclei occur during the metacystic stages. The amoebae may contain—if we exclude exceptional forms—any number of

nuclei from one to four; and as the nuclei are of two kinds (*N* and *n*), 14 kinds of amoeba are theoretically possible, namely:—

4-nucleates—*NNNN*,	*NNNn*,	*NNnn*,	*Nnnn*,	*nnnn* = 5
3-nucleates—*NNN*,	*NNn*,	*Nnn*,	*nnn*	... = 4
2-nucleates—*NN*,	*Nn*,	*nn*	...	... = 3
1-nucleates—*N*,	*n*	...	...	... = 2
				Total = 14

Of these possible combinations, two are absent from my preparations. These are the forms with three cystic nuclei (*NNN*) and with four daughter-nuclei (*nnnn*). If such forms occur, they must be excessively rare. The occurrence of the former[1] is so improbable that it may safely be ignored: the latter—if found—could hardly be distinguished from a young *NNNN* amoeba. Consequently, 12 different kinds[2] of metacystic amoebae are to be found, and the ancestry and progeny of each of these must be accounted for. This may be done as follows:

I. Quadrinucleate Amoebae.

Of these there are four kinds—*NNNN*, *NNNn*, *NNnn*, *Nnnn*.

(1) *NNNN* = 4-NUCLEATE OCTOGEN (Pl. XXIII, figs. 36, 37). Ubiquitous. Derived from cyst. Division[3] of one nucleus (*N*) gives *NNNnn*, which may be distributed by

(*a*) *Bipartition* into
NNNn and *n* (Pl. XXIII, fig. 47) ..... (i).
NNn and *Nn* (Pl. XXIII, figs. 44–46) ..... (ii).

(*b*) *Tripartition* into
Nn and *Nn* and *N* (Pl. XXIII, figs. 54, 55) .....(iii).

(*c*) *Quadripartition* into
NN and *N* and *n* and *n* (Pl. XXIII, fig. 58) .....(iv).

(2) *NNNn* = 4-NUCLEATE HEPTAGEN (Pl. XXIII, fig. 38). Common. Derived from I (1) (*a*) (i). Division of one nucleus (*N*) gives *NNnnn*, which may be distributed by *bipartition* into
NNnn and *n* (Pl. XXIII, fig. 52) ..... (i).
NNn and *nn* ([products] Pl. XXIV, figs. 59, 73) ..... (ii).
Nnn and *Nn* (Pl. XXIII, fig. 53) .....(iii).

[1] It is obvious that such an organism could arise in only one way—by tripartition of a quadrinucleate octogen (*NNNN*) into *n*, *NNN*, and *n*. Tripartitions at this stage are rare, in any case: and a division of this sort, if found, could only be regarded as a freak.

[2] If the 5-nucleate form (*NNNnn*) already noted—which is rare but constant—be included, then there are actually 13 kinds (excluding aberrant forms).

[3] Non-division of the cytoplasm results in the formation of a 5-nucleate octogen (*NNNnn*), as already noted. The development of these forms is described below (see p. 399).

(3) *NNnn* = 4-NUCLEATE HEXAGEN (Pl. XXIII, fig. 39). Uncommon. Derived from I (2) (i). Division of one nucleus (*N*) gives *Nnnnn*, which may be distributed by *bipartition* into

Nnnn and *n* (Pl. XXIII, fig. 57) ..... (i).
Nnn and *nn* ([products] Pl. XXIV, figs. 60, 73) ..... (ii).
Nn and *nnn* ([products] Pl. XXIV, figs. 72, 61) .....(iii).

(4) *Nnnn* = 4-NUCLEATE PENTAGEN (Pl. XXIII, fig. 40). Very rare. Derived from I (3) (i). Division of the *N* nucleus would give *nnnnn*, which might be distributed by *bipartition* into

nnnn and *n* [not found] ..... (i).
nnn and *nn* ([possible products] Pl. XXIV, figs. 61, 73)..... (ii).
[I have not found these division-stages.]

II. **Trinucleate Amoebae.**

Of these there are three kinds—*NNn*, *Nnn*, *nnn*.

(1) *NNn* = 3-NUCLEATE PENTAGEN (Pl. XXIV, fig. 59). Abundant. Derived from I (1) (*a*) (ii) or I (2) (ii). Division of one nucleus (*N*) gives *Nnnn*, which may be distributed by

(*a*) *Bipartition* into
Nnn and *n* (Pl. XXIV, fig. 66) ..... (i).
Nn and *nn* (Pl. XXIV, fig. 65) ..... (ii).

(*b*) *Tripartition* into
N and *nn* and *n* (Pl. XXIV, fig. 67) .....(iii).

(2) *Nnn* = 3-NUCLEATE TETRAGEN (Pl. XXIV, fig. 60). Uncommon. Derived from I (2) (iii); I (3) (ii); or II (1) (*a*) (i). Division of one nucleus (*N*) gives *nnnn*, which may be distributed by

(*a*) *Bipartition* into
nnn and *n* ([products] Pl. XXIV, figs. 61, 84) ..... (i).
nn and *nn* (Pl. XXIV, fig. 70) ..... (ii).

(*b*) *Quadripartition* into
n and *n* and *n* and *n* (Pl. XXIV, fig. 71) .....(iii).
[Tripartition also possible, but not found.]

(3) *nnn* = 3-NUCLEATE TRIGEN (Pl. XXIV, fig. 61). Extremely rare. Derived from I (3) (iii) or I (4) (ii) or II (2) (*a*) (i). I have found these forms, but have not succeeded in tracing their development.
[Their division might produce by

(*a*) *Bipartition*
nn and *n* ([products] Pl. XXIV, figs, 73, 84) ..... (i).

(*b*) *Tripartition*
n and *n* and *n* ([products] Pl. XXIV, fig. 84) ..... (ii).
but such divisions I have not actually seen.]

III. **Binucleate Amoebae.**

Of these there are three kinds—*NN*, *Nn*, *nn*.

(1) *NN* = 2-NUCLEATE TETRAGEN (Pl. XXIV, fig. 74). Extremely rare. Derived only—so far as I have ascertained—from I (1) (*c*) (iv); though other origins are possible. Division of one nucleus (*N*) gives *Nnn*, which may be distributed by

(*a*) *Bipartition* into
Nn and *n* [not found] ..... (i).

(*b*) *Tripartition* into
n and *n* and *N* (Pl. XXIV, fig. 81) ..... (ii).

[I have found a single *NN* specimen in which both nuclei are dividing simultaneously (Pl. XXIV, fig. 80); but I have been unable to obtain any later stage. The product would obviously contain four *n*-nuclei, and eventually give rise to four amoebulae: but how this is effected I do not know. I imagine that a simultaneous fission into four (as in fig. 71) would be the most likely result.]

(2) *Nn* = 2-NUCLEATE TRIGEN (Pl. XXIV, fig. 72). Very common. Derived from I (1) (*a*) (ii); I (1) (*b*) (iii); I (2) (iii); I (3) (iii); II (1) (*a*) (ii); or [?] III (1) (*a*) (i). Division of *N* gives *nnn*, which may be distributed by

(*a*) *Bipartition* into
nn and *n* (Pl. XXIV, figs. 75–78) ..... (i).

(*b*) *Tripartition* into
n and *n* and *n* (Pl. XXIV, fig. 79) ..... (ii).

(3) *nn* = 2-NUCLEATE DIGEN (Pl. XXIV, fig. 73). Common. Derived from I (2) (ii); I (3) (ii); [?] I (4) (ii); II (1) (*a*) (ii); II (1) (*b*) (iii); II (2) (*a*) (ii); [?] II (3) (*a*) (i); or III (2) (*a*) (i). These organisms always divide—without any previous nuclear division—into two (*n* and *n*). See Pl. XXIV, figs. 82, 83. [Note *resting* nuclei: cf. fig. 92, Pl. XXV.]

IV. **Uninucleate Individuals.**

Of these there are two kinds—*N* and *n*.

(1) *N* = 1-NUCLEATE DIGEN (Pl. XXIV, fig. 86). Uncommon. Derived (by multiple fission only) from I (1) (*b*) (iii); I (1) (*c*) (iv); II (1) (*b*) (iii); or III (1) (*b*) (ii). These amoebae divide, like ordinary trophic forms, into two amoebulae by bipartition (Pl. XXV, figs. 87–92). From trophic amoebae they are distinguishable only by their smaller size (cf. figs. 87–92 (Pl. XXV) with (Pl. XXII) figs. 8–14).

(2) *n* = 1-NUCLEATE AMOEBULA (Pl. XXIV, fig. 84). Ubiquitous. The final product of all metacystic development, variously derived from I (1) (*a*) (i); I (1) (*c*) (iv); I (2) (i); I (3) (i); [?] I (4) (i); II (1) (*a*) (i); II (1) (*b*) (iii); II (2) (*a*) (i); II (2) (*b*) (iii); [?] II (3) (*a*) (i) and II (3) (*b*) (ii); [?] III (1) (*a*) (i); III (1) (*b*) (ii); III (2) (*a*) (i); III (2) (*b*) (ii); III (3); or IV (1).

All the ordinary metacystic forms discovered or discoverable in cultures have now been accounted for, but it remains to consider certain aberrant individuals.

Quinquenucleate Amoebae.—As already noted, these occur regularly, but are rare. Almost invariably they have the nuclear constitution *NNNnn* (= 5-nucleate octogen, Pl. XXIII, fig. 41), and arise by division of one nucleus—without cytoplasmic fission—of an ordinary *NNNN* amoeba (cf. Text-fig. B, 4; p. 395). Such organisms develop essentially like quadrinucleate octogens: but in so doing they may produce types of amoebae not yet described.

Theoretically, *NNNnn* may give—by division of one *N*—six nuclei (*NNnnnn*); and these may be distributed, by bipartition, in the following five ways:

NNnnn and *n*	..... (i).
NNnn and *nn*	..... (ii).
NNn and *nnn*	.....(iii).
Nn and *Nnnn*	.....(iv).
Nnn and *Nnn*	..... (v).

All these products would be indistinguishable from those formed in the usual ways, with the exception of the 5-nucleate form *NNnnn* (i). This—a quinquenucleate heptagen—ought to occur, therefore, though very rarely. I have succeeded in finding it several times (Pl. XXIII, fig. 51, shows one specimen): and I have also two specimens which demonstrate how it is formed (Pl. XXIII, figs. 49, 50; see method (i) above).

The later development of such an amoeba would theoretically involve the occasional formation of yet another kind of 5-nucleate—a hexagen, *Nnnnn*—by bipartition. (All other products would be identical with forms already described.) I have found one such organism only (Pl. XXV, fig. 94). It does occur, therefore, and its occurrence fulfils the theoretical expectation.

Of 5-nucleate amoebae, consequently, there are three kinds—*NNNnn*, *NNnnn*, *Nnnnn*. No others are theoretically required[1] or discoverable in my cultures.

I have, moreover, a single specimen (Pl. XXIII, fig. 56, which I illustrate as a curiosity) showing, apparently, the multiple fission of a 5-nucleate octogen (*NNNnn*), following simultaneous division of two (*N*) nuclei, into four daughter-individuals—*N*, *nn*, *nn*, *nn*.

Sexnucleate Amoebae.—I have found a few amoebae containing six nuclei. They have the constitution *NNnnnn* (sexnucleate octogen, Pl. XXV, fig. 96), and hatch out of 6-nucleate cysts[2] (fig. 95). They are extremely rare, and I have not succeeded in tracing their development further.

I have also found two 6-nucleate amoebae with the constitution *NNNNnn* (fig. 97). They are possibly products of an 8-nucleate (see below): but I do not know how such forms arise or how they evolve.

[1] A form *nnnnn* might conceivably arise by binary fission of an *Nnnnn* amoeba: but considering the rarity of its parent it is hardly to be expected. I have never found it.

[2] Cf. p. 382 *supra*.

Septemnucleate Amoebae.—I have found one such organism only (Pl. XXV, fig. 99). It has the constitution *Nnnnnnn*, and might be produced by the simultaneous division of three nuclei—without cytoplasmic fission—in an *NNNN* amoeba. I have one specimen showing this process (Pl. XXV, fig. 98): but I know nothing else about the origin or fate of such amoebae.

Octonucleate Amoebae.—A few of these occur in every culture made from culture-cysts. Their nuclei are all of the same size. Fig. 93 (Pl. XXV) is a typical example. They hatch out of 8-nucleate (supernucleate) cysts[1], in this state, and subsequently feed and grow: but although I have made many attempts to do so, I have never been able to ascertain their later history. They disappear from normal cultures on continued incubation; but whether they ultimately divide or die I do not know.

Amoebae with more than eight nuclei do not occur normally in the metacystic development of Strain *K*. 28 c or any other strain which I have studied.

The time usually occupied in the whole metacystic development—from the moment of hatching to the final production of all eight amoebulae—is variable. The daughter-amoebae cannot all be watched continuously, and their nuclear constitution cannot be accurately determined during life. Nevertheless, the average course of development can be approximately ascertained by studying and counting the various forms present in cultures of different ages. With Strain *K*. 28 c the first division usually takes place about two hours after hatching; and the metacystic development is normally completed in 11–13 hours —reckoning from the beginning of incubation[2].

With the formation of uninucleate amoebulae the metacystic development is at an end. These little organisms feed and grow, and finally become ordinary trophic amoebae which develop in the manner already described. They reach their full size in a few hours, and are young amoebae—not gametes or conjugants. As I have watched them for many hours, and studied them carefully and continuously at all times, I make this statement with considerable confidence. The close approximation of the nuclei in metacystic organisms is, at first sight, suggestive of a nuclear fusion: but this never occurs. Amoebae with two adherent nuclei (binucleate trigens (*Nn*) especially), when seen for the first time, might easily be mistaken for stages in a gametic union: but I have ascertained the origins of these forms and their later development, and I have no doubt whatsoever that neither these nor any other metacystic products are concerned with any sexual process[3].

In confirmation of this interpretation, I may add that in the nuclear divisions, at all stages in the life-cycle, I find no evidence of chromosomic re-

[1] See fig. 22 (Pl. XXII), and cf. p. 382 *supra*. I have witnessed the hatching of these cysts.

[2] This is not necessarily true of all strains. For instance, my Strains *DKB*. and *DMK*. both develop more slowly under identical conditions—corresponding stages occurring, as a rule, 1–2 hours later than in *K*. 28 c.

[3] Other workers, I am aware, have already made similar statements: but as no worker—to my knowledge—has yet seen all the metacystic stages, I can only regard these assertions as conjectural. They possess whatever value attaches to a negation made in ignorance of the facts.

duction. Although I am as yet unable to count the chromosomes with absolute certainty, I am satisfied that their number is approximately the same at all stages. No halving or doubling occurs in the trophic amoebae, the cysts, or the metacystic organisms.

I have therefore no evidence of a "sexual cycle" at any stage, but much evidence to show that the whole life-history of *E. histolytica* is sexless.

IV. DISCUSSION.

Now that I have described my findings in some detail, I can discuss their bearing upon those previously recorded by others. But I shall here consider essential points only, because detailed analysis seems to me either impossible or unnecessary[1].

The only author who claims to have seen the whole life-history of *E. histolytica* outside its host is, so far as I am aware, K. Yoshida (1918, 1920)[2]. I am quite unable to understand this Japanese worker's papers; and most of the "development" which he describes and figures appears, to me, to be a misinterpretation of degenerative stages observed under peculiar and highly abnormal conditions. At all events, his complex "life-cycle"—with its unique "sexual" and other stages—is unlike anything that I have ever seen. Moreover, he was unable to cultivate his amoebae continuously (if at all); and the development which he describes took place at 27° to 30° C.—a temperature at which the strains which I have studied will not develop. In view of this, and many other radical discrepancies between Yoshida's findings and my own, I am unable to regard his claim as justified. In any case, he did not obtain the whole life-cycle of *E. histolytica* in his "cultures," but at most—if his confusing account can be credited—induced amoebae (from man) to encyst, and cysts (also from man) to hatch, *in vitro*.

Darling (1913) had previously described what he regarded as a development of the cysts—including excystation—outside the body. His experiments were made with cysts (from human stools) kept in moist chambers (apparently at room-temperature, since no mention is made of any incubation); but the phenomena observed were obviously, in my opinion, mostly degenerative. After many trials I have never seen anything but the degeneration and death of cysts studied by similar methods; and Darling's incomplete account of the formation of four small amoebae inside the cyst—which subsequently escape one at a time—is, to my mind, manifestly erroneous[3].

Penfold, Woodcock, and Drew (1916) have described and illustrated the excystation of *E. histolytica* (cysts from human stools) in a medium composed

[1] The early history of our knowledge of the development of *E. histolytica* I have already discussed elsewhere (Dobell, 1919).

[2] "The two papers, in which different culture media are described, together present the entire developmental cycle of *Entamoeba tetragena in vitro*" (Yoshida, 1920, p. 374).

[3] Yorke and Adams (1926, pp. 279–280) consider that too little attention has previously been paid to these observations; but as they believe that their own "suggest that Darling was mistaken" (*ibid.*, p. 288), it is not clear to me why they attach so much importance to his apparent errors.

of weak broth and *liquor pancreaticus*. According to their account, hatching occurs after incubation for 5–6 hours. The protoplasm first "becomes very slightly retracted from the cyst-membrane," and then changes shape "very slowly"—being only "slightly amoeboid." "Dissolution of the cyst-membrane" is said then to take place "over a small area only": but the illustration (their Fig. 8) shows that the diameter of the hole so formed is equal to about one-eighth of the circumference of the cyst. Through this large breach the whole of the protoplasm "gradually flows out of the cyst." In the figure the hatched amoeba (Fig. 8) is seen to contain a large chromatoid body. In all these details, therefore, the observations of these authors differ entirely from mine. I have never been able to cause excystation by the method indicated[1]: I have never seen a cyst hatch before its chromatoids had disappeared: and the very active movements inside the cyst prior to hatching, and the escape of the amoeba through a minute pore in the way I have described, are wholly unlike anything in this description.

In one respect, however, the account given by these authors agrees with my observations. They state that the protoplasm emerges from the cyst as a single amoeba—not as four amoebulae. Nevertheless, they were unable to determine how many nuclei their excysted organism contained[2], or to follow its development further. Their attempts at cultivation all failed.

Although the interpretation of these observations still appears to me problematic, it is at least clear that they supply no conclusive proof that Penfold, Woodcock, or Drew, ever witnessed the normal process of excystation in *E. histolytica*.

Two years after these observations had been recorded, Cutler (1918) announced that he had succeeded in cultivating *E. histolytica*; and also (1918, 1919) that he had been able to induce its encystation *in vitro*. His methods of cultivation, however, have failed entirely in my hands[3]; and in my experiments and those of others no encystation has ever occurred in cultures subjected to the treatments he described[4].

Cutler (1919) also stated that he had succeeded in causing *E. histolytica* to excyst *in vitro*. On repeating the experiment of Penfold, Woodcock, and Drew, he found that a small proportion of the cysts hatched, but "all the excysted amoebae were uninucleate[5]"; and he concluded that the excystation induced by this method was a pathological process. He then tried treating cysts with *liquor pepticus* followed by *liquor pancreaticus*, and found that "a very large proportion of the treated cysts react[6]. Of the excysted amoebae some are uninucleate...but the majority are quadrinucleate, Plate VII,

[1] See Dobell (1919), p. 58.

[2] Yorke and Adams (1926, p. 288) are in error when they state that Penfold, Woodcock, and Drew observed that the excysted organisms are quadrinucleate.

[3] See Dobell (1919), p. 70; Dobell and Laidlaw (1926 *a*), p. 295.

[4] See Boeck and Drbohlav (1925), p. 397; Dobell and Laidlaw (1926 *a*), pp. 298, 299.

[5] Cutler (1919), p. 138.

[6] I do not know whether this means "hatch," or what exact reaction is intended.

fig. 39." (This illustration shows an amoeba—apparently some 15 μ in diameter—with four well separated nuclei.) Cutler adds: "These forms I consider represent those which would emerge from the cyst under normal conditions"; but though they moved actively, and he watched them for five or six hours, he was not able "to observe them take any food or to determine definitely what is the next phase of their life-cycle." Nevertheless, he says "there is evidence [not given] that these quadrinucleate amoebae ultimately divide to form four small amoebulae, such as shown in Plate VII, fig. 40." (This figure shows a very minute uninucleate organism, apparently about 4 μ in diameter.)

At first sight it looks as though Cutler really observed the hatching of the cyst, and the liberation of a single 4-nucleate amoeba: but the added details do not support this interpretation, and make his observations extremely difficult to understand. Concerning the actual process of excystation he says nothing; and the newly hatched metacystic amoeba, with its characteristic clumped nuclei and voracious appetite, is quite unlike his organisms with discrete nuclei and no desire to feed. Moreover, his "amoebula" believed to result from such a parent is far smaller than any metacystic form that I have ever found, and the metacystic development is not—as he supposed—a division into four. Cutler's observations, therefore, appear to me—in the light of my own subsequent findings—as problematic as those of his precursors[1].

The first paper in which I have found any observations which accord with my own, and which therefore require no ingenuity on my part to interpret, is that of Chatton (1917). He did not, it is true, cultivate *E. histolytica* or study its development *in vitro*: but he succeeded in obtaining excystation *in vivo*, and his observations seem to me so important that I must mention them here.

Chatton (1917) fed kittens on ripe cysts of *E. histolytica* (from human faeces), and then attempted to study the early stages in the life-cycle by killing his animals at various intervals and examining their intestinal contents. He found that the chromatoid bodies rapidly disappeared from the ingested cysts, and that these then hatched in the small intestine (after incubation *in vivo* for $3\frac{1}{2}$ to $4\frac{1}{2}$ hours). No nuclear changes occurred in the cysts, however, and their nuclei remained separate. But when the cysts hatched, they liberated quadrinucleate amoebae whose nuclei approached one another and became agglomerated "*en tetraèdre*": and these organisms were seen to be actively motile and phagocytic—ingesting the bacteria in the gut-contents.

This description agrees in detail with what I have observed in cultures; and Chatton's drawings (his figs. 7–9) are such faithful pictures of recently excysted amoebae, that I can no longer doubt[2] that he really saw the first stages

[1] Cutler (1919) does not refer to Chatton's earlier publication (1917), and was apparently ignorant of it when he wrote—unless he included it in his general condemnation of French observations (1919, p. 130).

[2] I formerly doubted whether these stages were normal (Dobell, 1919, p. 57)—chiefly because Chatton obtained no further development in his kittens, and was unable to establish infection in them. The ingestion of bacteria and the clumping of the nuclei also appeared to me abnormal. I had myself previously attempted similar experiments (in 1916), which failed through ill-luck or unskilfulness on my part.

in the metacystic development of *E. histolytica*. But he was unable to follow it further, and he never saw the actual process of excystation. He believed, on the contrary, that the amoebae were freed by the gradual dissolution of the cyst wall[1]. Despite these shortcomings, however, Chatton's paper appears to me to record the first accurate observations ever made upon the metacystic development of *E. histolytica*.

The observations of Boeck and Drbohlav have already been referred to. In 1924 they succeeded—probably for the first time—in cultivating *E. histolytica*: and they probably were the first to obtain its encystation *in vitro*[2], while they also—in all probability—were the first to obtain a culture by hatching cysts from human stools[3]. But they did not obtain the whole life-cycle of the parasite in their cultures, and they were unable to observe encystation, excystation, or any stages in the metacystic development.

So far as I am aware, the only account yet published of the excystation and metacystic development of *E. histolytica* in cultures is that of Yorke and Adams (1926). They worked with cysts from human stools—often concentrated[4], apparently, by the specific-gravity method devised by Yoshida (1920)—and cultivated their amoebae in the medium of Boeck and Drbohlav: they found that cultures could be obtained by incubating cysts in this medium (in agreement with the observations of Boeck and Drbohlav and of myself and Laidlaw): and in their cultures they obtained active amoebae with four adherent nuclei—like those discovered by Chatton. All these observations are thus easily reconcilable with those made previously; but when the other findings of Yorke and Adams are examined more closely, they are by no means so easy to understand.

According to these authors, "it can be laid down as a general rule that in pre-excysting forms...nuclear agglomeration can always be seen" (p. 290); and this is clearly shown in all their figures[5]. (This rule is certainly not obeyed by Strain *K*. 28 c.) The amoeba then escapes from the cyst through a large irregular tear[6] in its wall. This process of excystation is unlike anything that

[1] He believed that he saw "Amincissement notable de l'enveloppe qui perd sa réfringence et son double contour et finalement disparaît, laissant à nu un corps sphérique encore immobile. Les pseudopodes ne se forment qu'un certain temps après la dissolution de l'enveloppe" (Chatton, 1917, p. 835).

[2] Cf. Boeck and Drbohlav (1925), p. 393.

[3] *Ibid.*, p. 385.

[4] But apparently without taking the very necessary precaution of freeing the cysts from concomitant trophic forms. Yoshida did this by treating them with strong hydrochloric acid.

[5] Yorke and Adams (1926), Pl. XXII, 1–8. They say, however, that "the nuclei in the living individual can be distinguished only with the greatest difficulty" (p. 286), though they succeeded in drawing them with the *camera lucida*: but the nuclei depicted are far smaller than any I have ever seen at this stage—most of them measuring not much more than 1μ in diameter.

[6] Their actual words are: "a rent apparently occurs in the cyst envelope..." (p. 287); but that the rent itself is real, and not merely apparent, is evident from the further statement that "at times, by careful focussing and suitable illumination, it can be clearly seen (Pl. XXII, fig. 11)." This figure shows a huge jagged breach in the cyst wall.

I have ever seen; but Yorke and Adams say they witnessed it often[1], and this makes it very difficult to reconcile their observations with mine. Some other details which they note are, however, in agreement with my observations.

The views of Yorke and Adams regarding the metacystic development may be summarized in their own words. They say[2]: "We believe the usual fate of the excysted quadrinucleate *Entamoeba histolytica* is its subdivision into four uninucleate amoebae, either by first dividing into two binucleate individuals, each of which again subdivides into two uninucleates, or probably most commonly by throwing off uninucleates one at a time." This belief, as will be evident from my foregoing account of the metacystic development, is opposed to mine in every detail; and I can find nothing to justify it in the figures published[3]. No actual stages in division are described or illustrated; and most of the metacystic amoebae which are depicted I am unable to identify with certainty, because the necessary nuclear details are not shown[4].

I do not understand how Yorke and Adams arrived at these conclusions, and other details which they record increase my perplexity. For example, they give[5] a series of counts of "vegetative" (metacystic, according to my nomenclature) amoebae—from an experiment "which was repeated many times" and "always gave similar results"—which is unlike any I have ever made[6]. Their metacystic forms also comprise certain giant organisms[7]—with 30–40 or even more nuclei, and which sometimes "constituted as much as 20 to 24 per cent. of the total amoebae"—whose origin and fate are not satisfactorily accounted for. Such amoebae do not occur normally in my cultures[8]; and I am unable to comprehend, from the description, the exact part which Yorke and Adams suppose them to play in the life-history.

There are very many other discrepancies between these observations and my own, but until the more important points are elucidated it seems profitless to discuss details. A detailed comparison between our divergent results is also difficult to make, because Yorke and Adams appear to have neglected nearly all the precautions which I regard as indispensable[9] in studying this subject.

It might perhaps be suggested that the disagreement in our observations is explicable by the fact that our material was different: since Yorke and Adams

[1] They say (p. 286) "On many occasions amoebae were actually observed to excyst." Consequently, their observation cannot be explained as a rare anomaly seen on an isolated occasion.

[2] *Op. cit.*, p. 291.

[3] *Op. cit.*, Pl. XXIII.

[4] Their figures were all drawn from specimens examined in iodine-solution; and I presume, therefore, that the originals are not available for further examination. Yorke and Adams do not appear to have studied any properly fixed and stained preparations.

[5] *Op. cit.*, Table III, p. 289.

[6] Cf. my Table, p. 389 *supra*.

[7] *Op. cit.*, Pl. XXIV.

[8] I have often found similar organisms in cultures of *E. histolytica* and *E. coli* developing under abnormal conditions—both in those initiated from cysts and in those made from trophic amoebae. They arise by repeated nuclear division, without cytoplasmic fission, and they ultimately degenerate and die. In my opinion such forms are monsters. They do not occur in the normal life-history of *E. histolytica*.

[9] See Section II, above—*passim*.

studied cysts from human stools, whereas I have worked with culture-cysts of simian origin. But this explanation will not suffice; for I have also studied natural cysts of *E. histolytica* from faeces, cysts from cultures of human strains, cysts of a human strain living in a monkey, and culture-cysts of the same strain (human from monkey). All these develop in essentially the same way, and none conforms with the descriptions of Yorke and Adams.

Excystation by the method which I have described occurs not only in *E. histolytica* but also in other entozoic amoebae and in some free-living forms. I have already made sufficient observations to warrant this statement, and additional evidence for it can be found in the works of others: for example, in Smith's (1927, 1928) papers on *Iodamoeba bütschlii*, in Hegner's (1927) incomplete observations on *E. coli*, and in Wilson's (1916) description of *Dimastigamoeba* (= *Naegleria*) *gruberi*. These workers do not interpret their observations exactly as I do, but the resemblance of their findings to mine is unmistakable.

If my account of the life-history of *E. histolytica* be confirmed and accepted, it will necessitate reconsideration of several protozoological problems in nomenclature, systematics, and pathology. I am fully aware of this, but shall not discuss these problems here. To my mind the time is not yet ripe for such discussion.

The development which I have seen and here imperfectly described occurs regularly in my cultures: but whether it is, or is not, identical with that which normally takes place in man has yet to be determined. Nevertheless, its regularity and its complexity, and the close agreement of many of its events with those already known to happen inside the human body, constrain me to believe that the phenomena which I have witnessed *in vitro* cannot be very different from those which normally occur—but cannot yet be seen—*in vivo*. I realize to the full, however, that this belief can be completely justified only by further accurate observation and much laborious experiment.

I have already demonstrated the process of excystation (in the living organism) and various stages of metacystic development to several friends and colleagues[1], but I shall be glad to show them to others also. Strain *K*. 28 c is still under cultivation, and I can supply living culture-cysts—for hatching and further cultivation—to anyone who wishes to verify my observations. At present I cannot give away or lend my permanent preparations, as I need them for other researches: but I will gladly demonstrate—by private appointment—any of the stages which I have described and figured to anybody who will take the trouble to visit my laboratory. It is not feasible to make such a demonstration in public.

I am confident that Strain *K*. 28 c will continue to develop in the way I have described. But it will do so only under the conditions specified, and

[1] These include Dr P. P. Laidlaw, F.R.S., Capt. S. R. Douglas, F.R.S., Dr A. Pringle Jameson, and Sir Charles Martin, F.R.S.

I therefore warn all who wish to repeat my experiments that they must be prepared to devote at least several months of continuous work to this study—with scrupulous observance of my directions—if they desire to see every developmental stage.

SUMMARY.

A pure strain of *Entamoeba histolytica* has been isolated and cultivated, and an attempt has been made to study and describe its whole life-history in detail.

This strain (*K*. 28 c) was derived from the dysenteric dejecta of a kitten experimentally infected *per os* by means of typical cysts from the faeces of a monkey (*Macacus sinicus*). It has now been under continuous cultivation for about 20 months (220 serial subcultures), and its development *in vitro* has been uniform throughout.

Methods have been devised, and are here described, whereby any desired stage in the life-history of this strain—amoebae, cysts, and all intermediate stages (including encystation and excystation)—can be readily procured *in vitro* at will.

Detailed study has shown that the trophic amoebae multiply in cultures by simple binary fission only, as they do in their natural hosts. Their mode of division is briefly described.

Encystation also occurs *in vitro* just as it does in the bowel, with formation of characteristic precystic amoebae and the final production of typical quadrinucleate cysts.

Excystation has been carefully studied, and it has been found that a single quadrinucleate amoeba escapes from each cyst through a minute perforation in its wall. An account is given of this remarkable process, which has not been described previously.

The 4-nucleate excysted (metacystic) amoeba has been found to produce a new generation of trophic forms by a complicated series of nuclear and cytoplasmic divisions, which are described in detail for the first time. The final result of this subdivision is the production of eight uninucleate amoebulae by each quadrinucleate amoeba hatched from a cyst.

These amoebulae are young trophic amoebae, and not gametes or conjugants. No sexual phenomena of any sort have been observed during the metacystic stages: and the life-history of *E. histolytica*, as visible *in vitro*, is thus wholly asexual.

A development similar to that here described in the case of Strain *K*. 28 c has been found to occur in many other cultivated strains of *E. histolytica*—including a strain isolated directly from man, and a human strain experimentally implanted in a monkey (*M. sinicus*) and recovered therefrom in pure culture. There are therefore good reasons for concluding that the development here described is not abnormal, and that it is probably closely parallel to that which occurs naturally inside man.

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DESCRIPTION OF PLATES XXII—XXV.

All figures on all Plates depict cultural forms of *Entamoeba histolytica* (Strain *K*. 28 c from *Macacus sinicus*), except figs. 1–4 which show the amoebae in their hosts of origin (monkey and kitten). All figures have been drawn from permanent preparations, with the aid of the *camera lucida*, at a uniform and exact magnification which, as here shown, is 2000 diameters (2 mm. = 1μ). The drawings have all been made under a Leitz 2 mm. apochromatic oil-immersion objective (N.A. = 1·40), with compensating oculars and a Leitz achromatic aplanatic condenser (N.A. = 1·40). Illumination was furnished by a "Pointolite" electric lamp with appropriate optical accessories.

The following abbreviations are used throughout to denote the methods of fixation and staining employed:

Bo. = Bouin's picro-formol-acetic fixative (aqueous).
S.A.A. = sublimate-alcohol fixative (Schaudinn's fluid) + 5 per cent. glacial acetic acid.
S.F. = sublimate-formol fixative (Carnoy and Lebrun).
Alc.FeHn. = alcoholic iron-alum haematein stain (C.D.).
Eo. = eosin (as counterstain).
Ethr. = erythrosin (counterstain).
FeClC. = iron-chlorocarmine stain (Hollande).
FeHx. = aqueous iron-alum haematoxylin (Heidenhain).

In the descriptions of excysting and metacystic stages, the time shown in parenthesis ( ) denotes the period for which the culture was incubated before the specimen illustrated was fixed. (Cf. p. 383 *supra*.) Numerals in square brackets [ ] are to be ignored[1].

These illustrations are meant to be taken in conjunction with the text, though they show much that is not there mentioned specifically. They are not diagrammatic, but careful representations of the actual specimens. Nuclear structures are always delineated completely; but the cytoplasm is usually shown in optical section, owing to the impossibility of exhibiting all inclusions clearly in a single drawing.

PLATE XXII.

Figs. 1, 2. Typical trophic amoebae from normal faeces of original simian host (*M. sinicus*). Note ingested bacteria. Bo. FeClC. Eo. [651]

Figs. 3, 4. Trophic amoebae from dysenteric discharge of experimentally infected kitten (No. 28). Note remains of three red blood-corpuscles in fig. 4. Bo. Alc.FeHn. Eo. [672]

Fig. 5. Typical trophic amoeba of Strain *K*. 28 c—isolated from Kitten 28—in culture (Boeck-Drbohlav egg-serum medium). Note ingested bacteria. S.A.A. Eosin-Toluidine blue. [727]

Fig. 6. Large trophic amoeba of same strain growing in Dobell-Laidlaw medium (horse-serum Ringer-eggwhite and starch). Note ingested bacteria and starch-grains (3). Bo. Alc.FeHn. [679]

Fig. 7. Medium-sized similar individual, packed with rice-starch. (Optical section: all ingested starch-grains not shown.) S.F. FeHx. [863]

Figs. 8–14. Successive stages in division (binary fission) of trophic amoebae, as seen in cultures. Bo. Alc.FeHn. [679]

Fig. 15. Newly divided daughter-individual. Bo. Alc.FeHn. [679]

Fig. 16. Precystic amoeba, from starch-culture. S.A.A. FeClC. Eo. [683]

Figs. 17–21. Successive stages in development of cysts in cultures. Fig. 17, Bo. FeClC. [822]; fig. 18, S.F. FeHx. Ethr. [862]; figs. 19, 20, S.A.A. FeHx. Ethr. [841]; fig. 21, S.A.A. FeClC. Eo. [683]

Fig. 22. Octonucleate (supernucleate) culture-cyst. Bo. Alc.FeHn. [692]

Figs. 23–33. Successive stages of excystation in cultures. (All 3 hours; except fig. 26, 3¾ hours.) Figs. 23–25, and 27–31, S.A.A. FeClC. [696]; fig. 26, S.A.A. FeClC. [701]; figs. 32, 33, Bo. Alc.FeHn. [689]

Figs. 34, 35. Recently excysted quadrinucleate amoebae. (3 hours.) S.A.A. FeClC. [696]

[1] They are the serial numbers of my permanent preparations in which the specimens depicted are contained, and are recorded merely for guidance in finding the actual objects again.

PLATE XXIII.

All figures on this Plate depict metacystic amoebae from preparations fixed with Bouin's fluid and stained with iron-chlorocarmine—unless a different technique is indicated. The terms used to describe these stages are those already employed in the text, where the notation is explained (p. 388).

Figs. 36–40 show the four kinds of quadrinucleate metacystic amoebae found in cultures.

Fig. 36. Quadrinucleate octogen (*NNNN*), with four small nuclei. Young form of amoeba shown in next fig. (7½ hours.) S.A.A. fixation. [720]

Fig. 37. Large 4-nucleate octogen (*NNNN*), with large nuclei. (6¾ hours.) [822]

Fig. 38. Quadrinucleate heptagen (*NNNn*). (6½ hours.) [820]

Fig. 39. Quadrinucleate hexagen (*NNnn*). (6¾ hours.) [822]

Fig. 40. Quadrinucleate pentagen (*Nnnn*). (6¾ hours.) [822]

Fig. 41. Quinquenucleate octogen (*NNNnn*). (6¾ hours.) [821]

Figs. 42, 43. Two stages of nuclear division in 4-nucleate octogens. (6¾ hours.) [821]

Fig. 44. Large 4-nucleate octogen (*NNNN*) dividing by bipartition into a binucleate trigen (*Nn*) and a trinucleate pentagen (*NNn*). (6½ hours.) [819]

Fig. 45. Later stage of the same process, in a smaller specimen. (7 hours.) [814]

Fig. 46. End-stage of the same. The products of such a division are shown in Pl. XXIV, figs. 72 and 59. (6¾ hours.) [821]

Fig. 47. Early stage in division of 4-nucleate octogen (*NNNN*) into a uninucleate amoebula (*n*) and a quadrinucleate heptagen (*NNNn*). The products of such a division are shown in Pl. XXIV, fig. 84, and in this Plate, fig. 38. (6¾ hours.) [822]

Fig. 48. Formation of a 5-nucleate octogen (*NNNnn*, fig. 41) by division of one nucleus of a 4-nucleate octogen (*NNNN*, fig. 43) without cytoplasmic fission. The two daughter-nuclei—in telophase—have run together instead of separating. (6¾ hours.) [822]

Fig. 49[1]. Nuclear division (spindle) in a 5-nucleate octogen (*NNNnn*). (6¾ hours.) [822]

Fig. 50. Division of 5-nucleate octogen (*NNNnn*) into a uninucleate amoebula (*n*) and a 5-nucleate heptagen (*NNnnn*). The products of such a division are shown in fig. 51 (this Plate) and fig. 84 (Plate XXIV). (7 hours.) [814]

Fig. 51. Quinquenucleate heptagen (*NNnnn*). (7 hours.) [813]

Fig. 52. Division of a 4-nucleate heptagen (*NNNn*) into a uninucleate amoebula (*n*) and a quadrinucleate hexagen (*NNnn*). The final products of such a division are shown in fig. 84 (Pl. XXIV) and fig. 39 (this Plate). (4 hours.) Bo. Alc.FeHn. [692]

Fig. 53. Division of a 4-nucleate heptagen (*NNNn*) into a 2-nucleate trigen (*Nn*) and a 3-nucleate tetragen (*Nnn*). The products of such a division are shown in figs. 72 and 60 (Pl. XXIV). (7 hours.) [816]

Fig. 54. Early stage in tripartition of a 4-nucleate octogen (*NNNN*) into a uninucleate digen (*N*), and two binucleate trigens (*Nn*). (7 hours.) [815]

Fig. 55. Later stage of same process. The products of such a division are shown in figs. 86 and 72 (Pl. XXIV). (6½ hours.) [820]

Fig. 56. Quadripartition of a 5-nucleate octogen (*NNNnn*) into three binucleate digens (*nn*) and one uninucleate digen (*N*), with simultaneous division of two *N*-nuclei. The products are shown in figs. 73 and 86 (Pl. XXIV). (7 hours.) [812]

Fig. 57. Division of quadrinucleate hexagen (*NNnn*) into a 4-nucleate pentagen (*Nnnn*) and a uninucleate amoebula (*n*). The products are shown in fig. 40 (this Plate) and fig. 84 (Pl. XXIV). (7 hours.) [814]

Fig. 58. Quadripartition of 4-nucleate octogen (*NNNN*) into two uninucleate amoebulae (*n*), one uninucleate digen (*N*), and a binucleate tetragen (*NN*). The products of such a fission are shown in figs. 84, 86, and 74 (Pl. XXIV). (6¾ hours.) [821]

[1] In the specimen from which this figure was drawn, two pairs of nuclei actually lie superposed, and they have been separated in the drawing—by slightly shifting the *camera-lucida* image—in order to show their structure more clearly.

PLATE XXIV.

All figures on this Plate depict metacystic amoebae from preparations fixed with Bouin's fluid and stained with iron-chlorocarmine—unless a different technique is indicated.

Figs. 59–61 show the three kinds of trinucleate metacystic amoebae found in cultures.

Fig. 59. Trinucleate pentagen (*NNn*). (6¾ hours.) [822]
Fig. 60. Trinucleate tetragen (*Nnn*). (6¾ hours.) [822]
Fig. 61. Trinucleate trigen (*nnn*). Stained FeHx. Ethr. (11 hours.) [785]
Fig. 62. Early stage (spindle) of nuclear division in trinucleate pentagen (*NNn*). (6¾ hours.) [822]
Figs. 63, 64. Later stages of same. (6¾ hours.) [821]
Fig. 65. Late stage of division (bipartition) of trinucleate pentagen (*NNn*) into a binucleate trigen (*Nn*) and a binucleate digen (*nn*). The products of such a division are shown in figs. 72 and 73. S.A.A. fixation. (7½ hours.) [720]
Fig. 66. Late stage of division (bipartition) of trinucleate pentagen (*NNn*) into a trinucleate tetragen (*Nnn*) and a uninucleate amoebula (*n*). The products of such a division are shown in figs. 60 and 84. (6½ hours.) [820]
Fig. 67. Late stage in tripartition of a trinucleate pentagen (*NNn*) into a uninucleate digen (*N*), a binucleate digen (*nn*), and a uninucleate amoebula (*n*). The products are shown in figs. 86, 73, and 84. (6½ hours.) [819]
Fig. 68. Nuclear division in a trinucleate tetragen (*Nnn*). (6½ hours.) [820]
Fig. 69. Later stage (telophase) of same process. (7 hours.) [812]
Fig. 70. Division (bipartition) of trinucleate tetragen (*Nnn*) into two binucleate digens (*nn*). The resultant individuals are illustrated in fig. 73. (7 hours.) [816]
Fig. 71. Quadripartition of trinucleate tetragen (*Nnn*) into four uninucleate amoebulae (*n*). (7 hours.) [813]

Figs. 72–74 illustrate the three kinds of binucleate metacystic amoebae found in cultures.

Fig. 72. Binucleate trigen (*Nn*). (6¾ hours.) [822]
Fig. 73. Binucleate digen (*nn*). (6¾ hours.) [822]
Fig. 74. Binucleate tetragen (*NN*). (6¾ hours.) [822]
Fig. 75. Nuclear division (spindle) in binucleate trigen (*Nn*). (7 hours.) [815]
Fig. 76. Later stage of same. (7 hours.) [810]
Fig. 77. Bipartition of binucleate trigen (*Nn*) into a binucleate digen (*nn*) and a uninucleate amoebula (*n*): daughter-nuclei in telophase. (7 hours.) [815]
Fig. 78. Final stage of same process. The products of such fission are shown in figs. 73 and 84. (7 hours.) [814]
Fig. 79. Tripartition of binucleate trigen (*Nn*) into three uninucleate amoebulae (*n*). (7 hours.) [816]
Fig. 80. Division of both nuclei in binucleate tetragen (*NN*). Stained FeHx. Ethr. (11 hours.) [785]
Fig. 81. Tripartition of binucleate tetragen (*NN*) into a uninucleate digen (*N*) and two uninucleate amoebulae (*n*). (7 hours.) [824]
Fig. 82. Bipartition (constriction) of binucleate digen (*nn*) into two uninucleate amoebulae (*n*). Note *resting* nuclei. (6½ hours.) [820]
Fig. 83. Later stage of same. (7 hours.) [814]
Fig. 84. Uninucleate amoebula (*n*). (7 hours.) [816]
Fig. 85. The same—larger individual with larger nucleus. (6¾ hours.) [822]
Fig. 86. Uninucleate digen (*N*). (6¾ hours.) [822]

PLATE XXV.

All figures, except fig. 95, depict metacystic amoebae from cultures. All are drawn—unless a different technique is indicated—from specimens fixed with Bouin's fluid and stained with iron-chlorocarmine.

Fig. 87. Uninucleate digen (*N*). (6¾ hours.) [822]
Figs. 88–91. Successive stages in nuclear division of uninucleate digen (*N*). Stained FeHx. Ethr. (11 hours.) [785]

Fig. 92. Final stage in bipartition of uninucleate digen (*N*) into two uninucleate amoebulae (*n*). (7 hours.) [816]

Fig. 93. Octonucleate amoeba—the product of an 8-nucleate cyst (fig. 22, Pl. XXII). (6½ hours.) [820]

Fig. 94. Quinquenucleate hexagen (*Nnnnn*). (6½ hours.) [821]

Fig. 95. Sexnucleate (supernucleate) cyst (*NNnnnn*). S.A.A. FeClC. [696]

Fig. 96. Amoeba with six nuclei (*NNnnnn* = sexnucleate octogen)—derived from a 6-nucleate cyst (fig. 95). (7 hours.) [817]

Fig. 97. Sexnucleate amoeba (*NNNNnn* = 6-nucleate decagen) of unknown origin. (6½ hours.) [820]

Fig. 98. Quadrinucleate octogen (*NNNN*) with three nuclei dividing simultaneously. (7 hours.) [816]

Fig. 99. Amoeba with seven nuclei (*Nnnnnnn*): ? product of preceding. S.A.A. fixation. (7½ hours.) [720]

(*MS. received for publication* 30. v. 1928.—Ed.)

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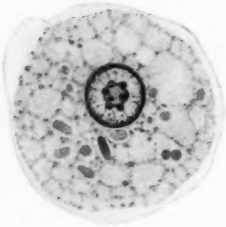

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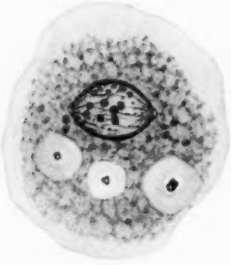

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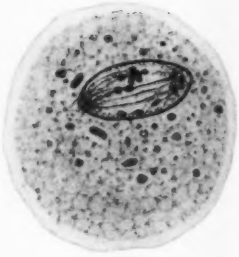

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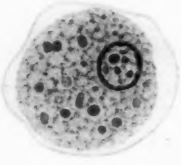

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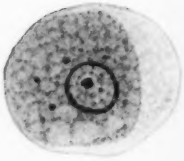

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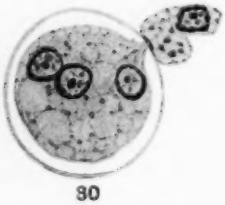

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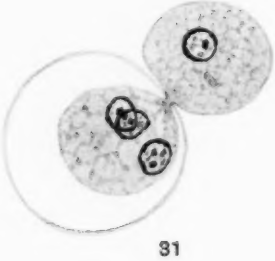

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PLATE XXII

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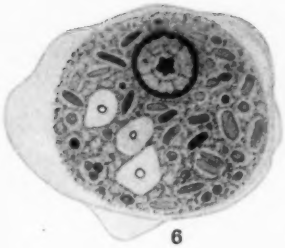

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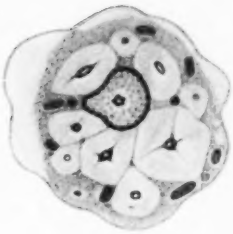

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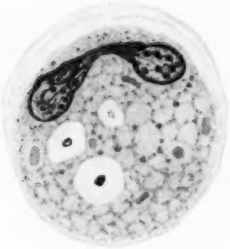

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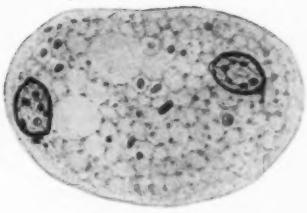

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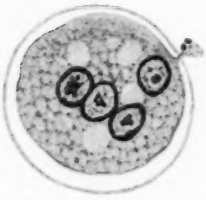

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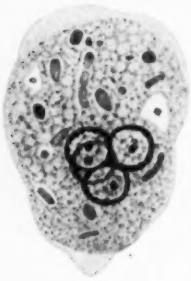

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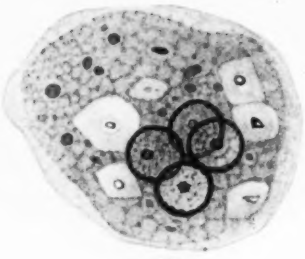

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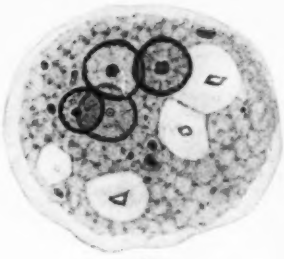

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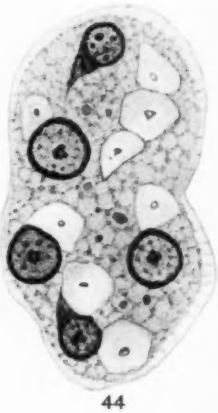

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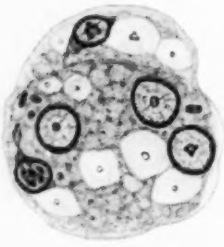

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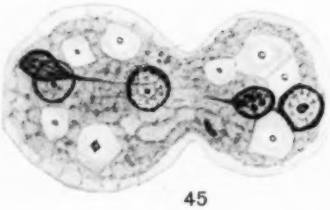

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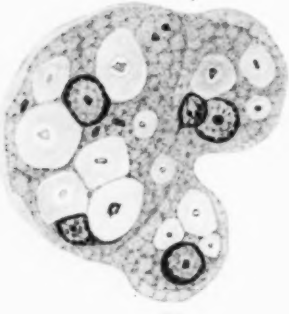

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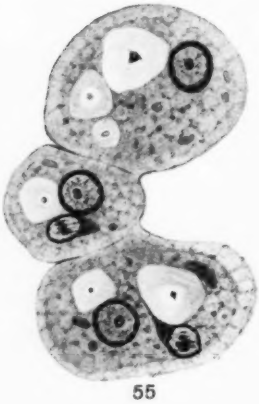

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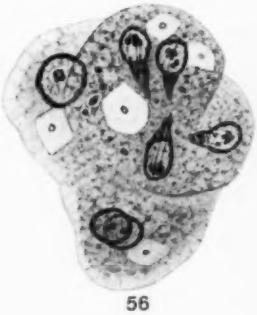

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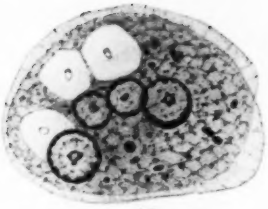

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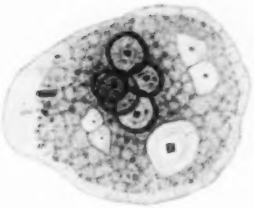

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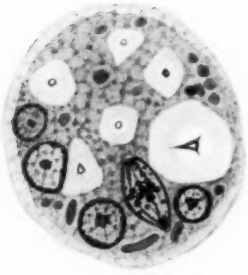

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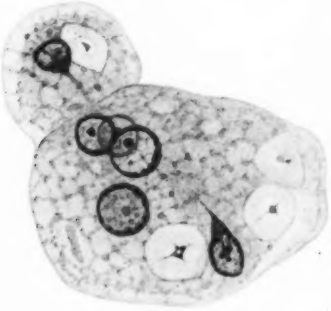

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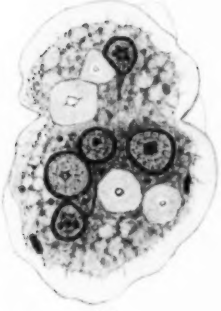

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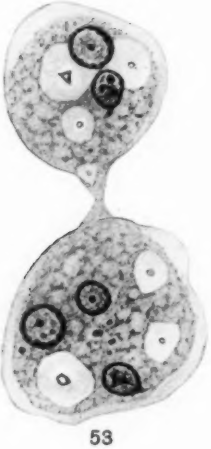

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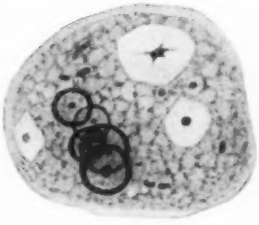

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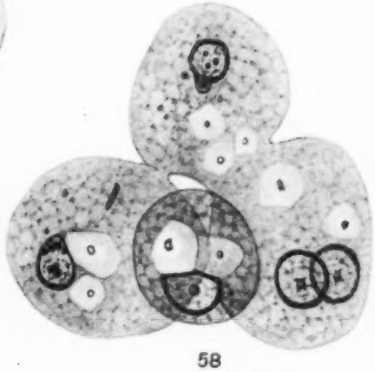

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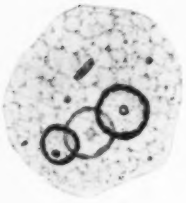

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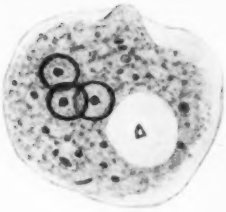

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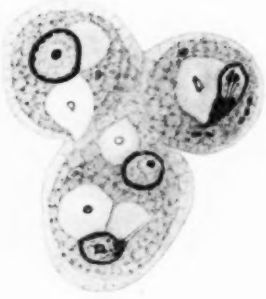

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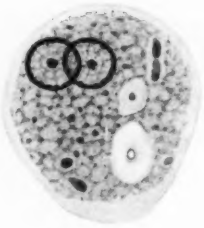

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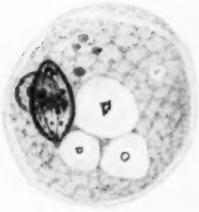

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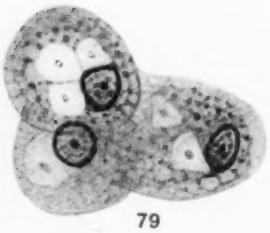

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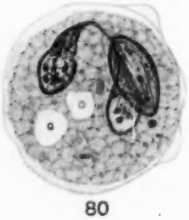

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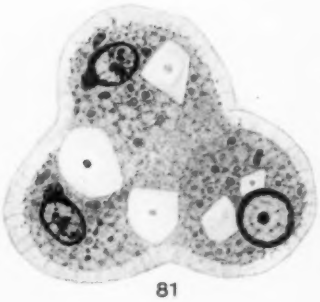

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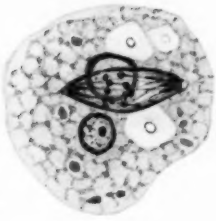

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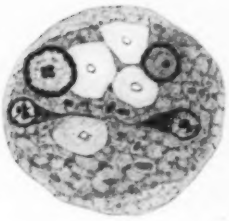

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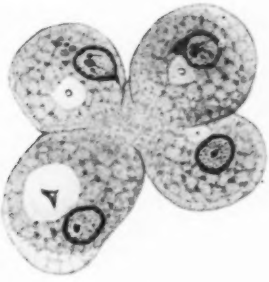

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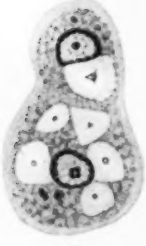

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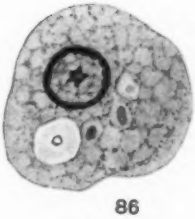

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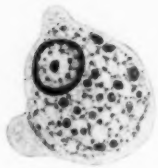

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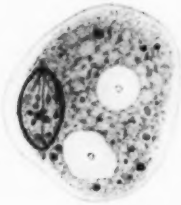

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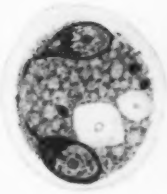

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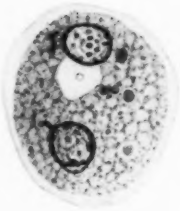

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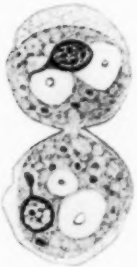

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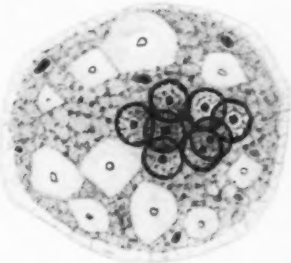

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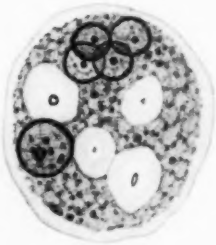

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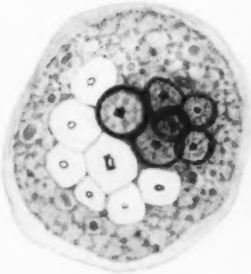

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STUDIES ON THE TREMATODE FAMILY STRIGEIDAE (HOLOSTOMIDAE). NO. XII. *AGAMODISTOMUM LA-RUÈI* SP. NOV.[1]

By R. CHESTER HUGHES.

University of Michigan, Ann Arbor, Michigan, U.S.A.

(With 3 Figures.)

Twelve larval trematodes, herein described as *Agamodistomum la-ruei* n.sp., were found by Prof. La Rue, at the autopsy of a racoon, *Procyon lotor lotor* (Linn.), which had been kept for several months in captivity near Douglas Lake, Cheboygan County, Michigan, during the spring and summer of 1925. The parasites were found by teasing the lungs apart in water. It was not determined whether they were in the alveoli of the lungs or in the tissue. They were apparently not encysted.

The specimens, fixed in sublimate acetic and stained in a dilute solution of Ehrlich's acid haematoxylin in 70 per cent. alcohol, were mounted ten *in toto* on one slide, and two in sections of 8 μ thickness on another[2]. The sections were counterstained with eosin. Living specimens were not studied.

Agamodistomum la-ruei closely resembles *A. marcianae*, which is parasitic in the garter snake, *Thamnophis marciana* (Baird and Girard), according to La Rue (1917), and in frogs, *Rana pipiens* Schreber and *R. clamitans* Latreille, according to Cort (1918). *A. la-ruei*, however, is somewhat smaller than *A. marcianae*, and since it occurs in a homothermous host it is probable that it belongs to a distinct species. Because of its similarity to *A. marcianae* as described by La Rue (1917, p. 3) it is unnecessary to give a detailed morphological description of *A. la-ruei*. The principal measurements of the ten specimens which were mounted *in toto* are shown in the accompanying table. A comparison of these measurements with those of *A. marcianae* (La Rue, 1917, p. 6) which are also of preserved specimens mounted *in toto* shows that the present species is consistently smaller. The salient features of *A. la-ruei* are shown in Figs. 1 and 2.

A comparison with type and cotype specimens (mounted *in toto* and in sections) of *A. marcianae*, which were kindly given to me for examination by

[1] Contribution from the Biological Station and the Zoological Laboratory, University of Michigan. This is the twelfth of a series of studies on the family Strigeidae by Prof. George R. La Rue, his students and associates.

[2] These slides bear Catalogue Nos. 278 and 279 in the parasite collection, Museum of Zoology, University of Michigan.

Prof. La Rue, shows that in general *A. la-ruei* differs as follows. The anterior end of the body is seldom constricted at the base of the oral sucker as it frequently is in *A. marcianae*, and the ducts of the penetration glands are somewhat larger in diameter and less coiled. These ducts are in general more sinuous, however, than Fig. 1 indicates.

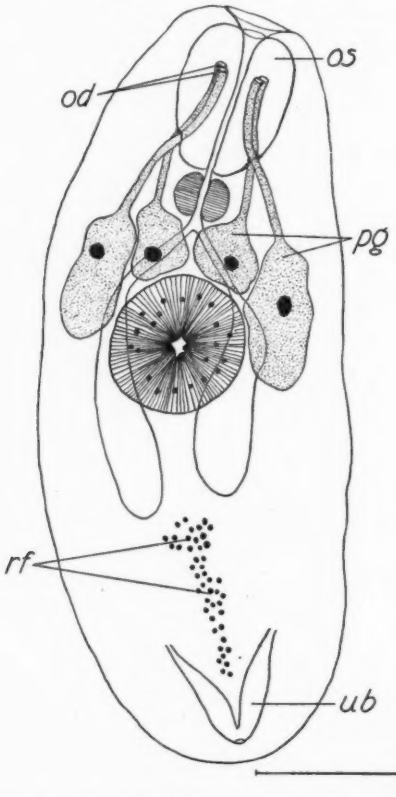

Fig. 1. *Agamodistomum la-ruei*, ventral view of a preserved specimen mounted *in toto*, drawn with the aid of a camera lucida. *od*, external openings of the ducts of the penetration glands; *os*, oral sucker; *pg*, penetration glands; *rf*, fundament of reproductive organs; *ub*, urinary bladder.

Measurements in Millimetres of Ten Preserved Specimens of Agamodistomum la-ruei *mounted* in toto.

		Minimum	Maximum	Average
Body	Length	0·275	0·353	0·307
	Width	0·148	0·190	0·164
Oral sucker	Length	0·056	0·081	0·069
	Width	0·045	0·056	0·051
Acetabulum	Length	0·056	0·070	0·064
	Width	0·063	0·074	0·068

DISCUSSION.

It has been shown by Cort (1918) and Cort and Brooks (1928, p. 199) that *Agamodistomum marcianae* develops from a strigeid cercaria. The larva, however, is hardly comparable to other strigeid metacercariae since after penetra-

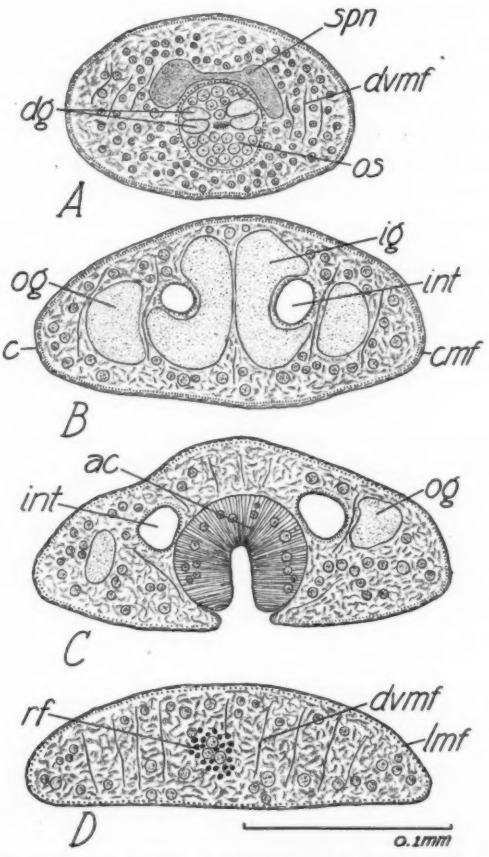

Fig. 2. *Agamodistomum la-ruei*, cross-sections drawn with the aid of a camera lucida: *A*, through oral sucker; *B*, between oral sucker and acetabulum; *C*, through acetabulum; *D*, through fundament of reproductive organs: *ac*, acetabulum; *c*, cuticle; *cmf*, circular muscle fibres; *dg*, ducts of penetration glands; *dvmf*, dorso-ventral muscle fibre; *ig*, inner penetration gland; *int*, intestinal caecum; *lmf*, longitudinal muscle fibres; *og*, outer penetration gland; *os*, oral sucker; *rf*, fundament of reproductive organs; *spn*, supra-pharyngeal nerve commissure.

tion into the second intermediate host it regenerates the penetration glands and, although it grows to much greater size, fails to undergo the profound metamorphosis common to most strigeids. These authors suggest that this agamodistome represents either an aberrant development brought about by

the penetration of the cercaria into an unsuitable host in which it is able to maintain itself and grow but not metamorphose, or the intercalation of an additional phase, involving an additional host, into the usual strigeid life cycle. The close similarity of *A. la-ruei* to *A. marcianae* suggests that it, too, may belong to the family Strigeidae. Descriptions and reports of other agamodistomes of similar structure occur in the literature. In fact not enough is known of these forms to preclude the possibility of general conspecificity. In the following synopsis these forms are all brought together on the supposition that they may all be strigeids. The parasites included in this synopsis are agamodistomes having in common a similar body form and four large penetration glands situated about the acetabulum. They differ from typical strigeid metacercariae in the complete absence of hind-body, hold-fast organ and reserve bladder.

AGAMODISTOMES SIMILAR TO *A. LA-RUEI* IN POIKILOTHERMOUS HOSTS.

1. **Agamodistomum tetracystis** (Gastaldi) mihi.

SYNONYMY: *Distoma tetracystis* Gastaldi (1854, p. 4).
Distomum tetracystis Ranae esculentae Gastaldi Diesing (1855 *a*, p. 64).
Distomum Tetracystis Gastaldi Diesing (1855 *b*, p. 390).
Distomum tetracystis Gast. Braun (1893, p. 871).

This form, parasitic in *Rana esculenta* in Europe, was briefly described by Gastaldi (1854, p. 4, Pl. 1, figs. 1–3). A description based on that of Gastaldi was published by Stossich (1889, p. 68). According to the figures and descriptions *A. tetracystis* differs from *A. la-ruei* and *A. marcianae* in that the oesophagus is long and slender and the pharynx is somewhat removed from the oral sucker. De Filippi (1855, p. 339) called attention to a strong similarity of *A. tetracystis* to *Cercaria microcotyla* de Filippi. Following this suggestion of de Filippi, Diesing (1855 *b*, p. 390) considered the two forms to be genetically related.

2. **Agamodistomum marcianae** (La Rue) Cort.

SYNONYMY: *Cercaria marcianae La Rue* La Rue (1917, p. 3).

This form was described in detail from preserved material by La Rue (1917, pp. 3–8, Pl. 1, figs. 1, 2). The spination and excretory system were studied in more detail in living specimens by Cort (1918, figs. 1, 2, 3), who also demonstrated that it was possible to introduce *A. marcianae* into garter snakes, *Thamnophis sirtalis sirtalis* (Linn.), by feeding the snakes with infected tadpoles. In this change of hosts the parasite shows no advance in development. Cort and Brooks (1928, p. 199) showed that *A. marcianae* develops from a strigeid cercaria, *Cercaria marcianae* (La Rue). *A. marcianae* has been found in *Thamnophis marciana* (Baird and Girard), *T. s. sirtalis* (Linn.), *Rana pipiens* Schreber and *R. clamitans* Latreille.

Notes and sketches pertaining to the distribution of *A. marcianae* in heavily infected tadpoles, prepared under the direction of Prof. La Rue by Mr Thomas Blair have been kindly given to me. These tadpoles had been

experimentally infected by Prof. W. W. Cort and Mr S. T. Brooks at Douglas Lake during the summer of 1925 and sent to Mr Blair in Ann Arbor where the following observations were made. The worms occur, free, chiefly in the subcutaneous lymph spaces and are most abundant in the ventral part of the abdomen (Fig. 3). A few were found in the deeper tissues. The specimens shown on the eyes (Fig. 3) were situated just beneath the skin covering the eye.

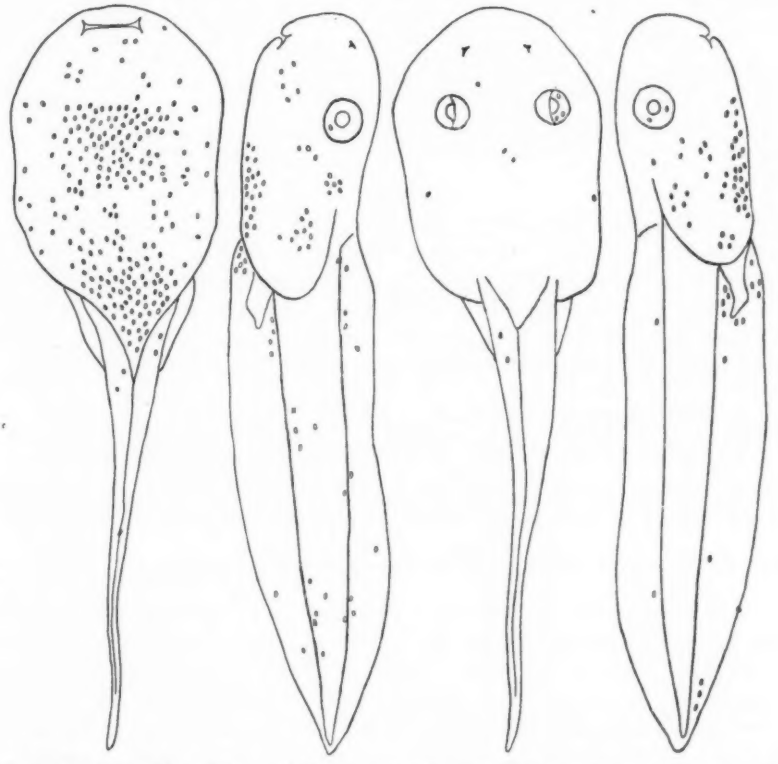

Fig. 3. A tadpole, *Rana pipiens* Schreber, heavily infested with *Agamodistomum marcianae* (La Rue). Four views (ventral, sinistral, dorsal, and dextral) showing the distribution of the parasites, chiefly in the subcutaneous lymph spaces. This tadpole contained (by count) 587 agamodistomes. Redrawn from sketches made with the aid of a camera lucida by Mr Thomas Blair.

3. **Agamodistomum ordinata** (Nicoll) mihi.

SYNONYMY: *Cercaria ordinata* Nicoll (1912, p. 767).

This species was described by Nicoll (1912, p. 767, text-fig. 107 A) from living specimens taken from three North American striped snakes which died in the Gardens of the Zoological Society of London. The conditions under which these snakes had been kept were not recorded and it is not possible to

determine whether they acquired their infections in England or in America. The agamodistome compares favourably in size and structure with *Agamodistomum marcianae* except that according to the figure the two penetration glands on either side pour their secretions into a common duct, whereas in *A. marcianae* each gland has a separate duct. These apparent differences may be due to an error in observation and it is possible that the two species are identical.

In Homothermous Hosts.

4. **Agamodistomum putorii** (Molin) Stossich.

Synonymy: *Distomum putorii* Molin (Molin 1858, p. 131).
Distomum putorii Molin (Braun 1893, p. 870).

Briefly described by Molin (1861, p. 224, Pl. V, fig. 4) this parasite was better described by von Linstow (1875, p. 192, Pl. II, fig. 5). A brief, but not original, description was published by Stossich (1892, p. 34). The parasite was found encysted in the muscles and on the oesophagus of *Foetorius putorius*. The similarity of this species with *Agamodistomum tetracystis* was observed by von Linstow. According to von Linstow's measurements *A. putorii* is considerably larger than *A. la-ruei*.

5. **Agamodistomum suis** Stiles.

Synonymy: *Distomeen in Schweinefleisch* Duncker (1881 *a*, p. 23).
Muskel-Distomeen Duncker (1881 *d*, p. 141).
Distomum sp. *Duncker* Braun (1893, p. 870).
Distomum musculorum suis Duncker (1896, p. 279).
Agamodistomum suis Stiles (1898, p. 28).
Leberegel aus dem Schweinefleisch Kästenbaum (1899, p. 244).

This parasite was first described and figured by Duncker (1881 *a*, p. 23, figs. 1–6) from specimens found by G. Leunis, a trichina inspector in Saxony. Other descriptions based on the original by Duncker have been published by Duncker (1881 *d*, p. 159, fig. 1; 1884, p. 39, figs. 1–6 and 1896, p. 278, figs. 1–8), Leuckart (1889, p. 154, fig. 86) and Stiles (1898, p. 28, fig. 1) and a figure by Kästenbaum (1899, fig. 33). Its occurrence has also been reported by Duncker (1881 *b*, p. 55; 1881 *c*, p. 154 and 1881 *c*, p. 141), Leuckart (1881, p. 46) and Schneidemuehl (1896, p. 302). The specimens reported from this country by Stiles (1900, p. 559) were not definitely identified with the present species. *Agamodistomum suis* is rare, being found only occasionally, encysted in the muscles of swine, *Sus scrofa domestica*, by European meat inspectors. According to measurements given by Stiles (1898) *Agamodistomum suis* is somewhat larger than *A. la-ruei*. According to the figure of Leuckart it differs from *A. la-ruei* in that the oral sucker is relatively shorter and the reproductive system is represented by three distinct fundaments.

Acknowledgements.

I wish here to express my grateful appreciation to Prof. George R. La Rue of the Zoological Laboratory and Director of the Biological Station, University of Michigan, by whom this work was suggested and under whom it has been conducted.

I am also indebted to Prof. La Rue for the use of the type specimens of *Agamodistomum la-ruei* and type and cotype specimens of *A. marcianae* all of which belong to his personal collection. My thanks are due to Mr Thomas Blair, of the Medical School of the University of Michigan, for the use of notes and sketches relative to the distribution of *Agamodistomum la-ruei* in tadpoles which had been experimentally infected by Prof. W. W. Cort and Mr S. T. Brooks.

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DEGENERACY IN PARASITIC COPEPODA IN RELATION TO THE DISTRIBUTION OF THEIR HOSTS IN TIME.

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I desire to call attention to a fact, which, though obvious enough to me, has not been mentioned by any author on parasitic Copepoda, viz. the relation of the degree of degeneration in these parasites to the distribution of their hosts in time.

(1) The lowest Phylum in the evolutionary series on which parasitic Copepoda have been found is the ANNULATA. But little is known of the Nereicolidae as represented by *Nereicola* on *Nereis*, but this genus appears to be a very degenerate form. The Saccopsidae as represented by the genera *Saccopsis* and *Xenocoeloma*, both parasites on different species of *Polycirrus*, (Terebellidae), are the most degenerate Copepod parasites known, having lost their appendages and alimentary tract. Moreover, the coelom of *Xenocoeloma* is continuous with that of the host; it is also hermaphrodite.

The Herpyllobiidae, a family consisting of seven valid genera and parasitic on the Polynoidae, is equally degenerate. All traces of segmentation and of articulated head- and neck-appendages have been lost, there is no mouth or alimentary canal, and the head has been converted into a voluminous organ buried in the tissues of the host—the only function of the head is to absorb nutritive solutions by endosmosis through expansions or rootlets. A full account of this family was given by me in 1926. Other parasites on Chaetopod worms are equally degenerate, *e.g. Bradophila* on *Brada*, *Trophoniphila* on *Trophonia*, discussed in the same paper (1926 *b*).

(2) The ECHINODERMATA are of as great, if not greater, antiquity than the Annulata. But little is known of the parasites of this Phylum, but they are enclosed in cysts or galls, and cut off from the external world except by a pore. K. Stephensen (1914–16) has described *Arthrochordeumium appendiculosum* (endoparasitic in the coelom of the proximal part of one of the arms of the Ophiurid *Astrocharis gracilis*), which is asymmetrical, shows but the faintest indications of segmentation and possesses only two pairs of appendages in both sexes, the antennules and maxillae being reduced to one-articled stumps. He also refers to four other genera. On the other hand, *Pionodesmotes phormosomae* Bonnier 1894 (occurring on the later Echinoid *Phormosoma uranus*), shows but little degeneration; the appendages are complete and

articulated whilst the body is segmented. The same remarks apply to the CANCERILLIDAE ectoparasitic on the cosmopolitan Ophiurid *Amphipholis squamata* (*vide* Stephensen, 1927).

(3) The next case affords the most notable exception to the view that I am advancing. The next Phylum in order of evolution is the ARTHROPODA, and parasitic on the CRUSTACEA is the family CHONIOSTOMATIDAE Hansen 1897. This family does not appear to show much degeneration. There is a loss of segmentation, but the appendages are complete and articled, though the antennules are small. Nevertheless, it is to be remarked that they are only parasites on the higher or Dekapoda members of the Crustacea and so may have more recently taken to parasitism, *e.g. Choniostoma* on *Hippolyte*, *Mysidion* on *Schizopoda*, *Aspidoecia* on *Erythrops*, *Stenothocheres* on Stenothoidae. Similarly *Nicothoë astaci* on *Astacus homarus* shows no degeneration, the antennules have as many as eleven articles (Leigh-Sharpe, 1926 *a*).

(4) The antiquity of the UROCHORDA is problematical, but there is no reason to believe that the Ascidians are a particularly ancient stock. The Copepods associated with them, the ASCIDICOLIDAE, are merely commensal and not parasites at all. They resemble free-living forms in habits and structure. The same remarks apply to the NOTODELPHYIDAE, *e.g. Doropygus trisetosus*, occurring on *Styela* (*vide* Stephensen, 1927).

(5) Since no parasitic Copepods have been reported on Cyclostomata, the oldest VERTEBRATA to bear parasites are the PISCES, and of these the most ancient are the ELASMOBRANCHII, to which the greatest number of the degenerate family LERNAEOPODIDAE are confined. This family consists of fixed parasites, permanently attached under the skin of the host by a bulla or button formed from the larval frontal filament with which the tips of the second maxillae are fused. The trunk seldom shows traces of segmentation; one or two segments may sometimes be demarcated anteriorly. The antennules have as few as four articles. The genera *Lernaeopoda*, *Lernaeopodina*, *Ommatokoita*, *Brianella*, *Charopinus* and *Thomsonella* are entirely confined to Elasmobranchs. There are in fact only two genera containing many species, viz. *Clavella* and *Brachiella*, that never occur on Elasmobranchs. *Tanypleurus* without appendages or segmentation and even more degenerate occurs on *Somniosus microcephalus*.

(6) In the TELEOSTOMI the CROSSOPTERYGII are the most ancient forms, and on *Polypterus*, several species, *Lernaea haplocephala* (Cunnington, 1914) has been found. The LERNAEIDAE to which *Lernaea* belongs are a degenerate family in which segmentation has been lost, the head and neck are buried in the host's tissues, but there has been little modification of the appendages. The larval swimming feet have been preserved, but the antennules have three articles.

(7) The HOLOCEPHALI are also an ancient stock, and on *Hydrolagus* [*Chimaera*] *colliei* Wilson has recently (1908) discovered *Chondracanthus epachthes*, a member of the degenerate family CHONDRACANTHIDAE.

(8) In the TELEOSTEI, the five groups that still have an open pneumatic duct from the air-bladder (PHYSOSTOMI) are the most ancient. In the SALMONICLUPEIFORMES, *Salmincola* and *Basanistes* of the family LERNAEOPODIDAE occur on *Salmo*, one species of *Clavellisa* on *Clupea*, and *Lernaeenicus sprattae* and *L. encrasicola* of the family LERNAEIDAE on the eye of *Clupea*, sprat and anchovy. In the CYPRINISILURIFORMES, ESOCIFORMES and ANGUILLIFORMES respectively species of *Lernaea* occur as follows: *L. cyprinacea* on *Cyprinus* and allied genera, *L. esocina* on *Esox*, and *L. elegans* on *Anguilla*, *Cyprinus* and allied genera (Leigh-Sharpe, 1925 *a*). The genus *Tracheliastes* (LERNAEOPODIDAE) occurs on *Cyprinus* and *Silurus*.

(9) The GADIFORMES which occupy a position intermediate between the Physostomi and the Acanthopterygii, have the genus *Clavella* of the Lernaeopodidae peculiarly their own (Leigh-Sharpe, 1925). Various species of *Gadus* are also hosts for *Lernaeocera* spp. and *Haemobaphes* of the Lernaeidae.

(10) The ancient TRIGLIFORMES harbour several species of *Brachiella* (Lernaeopodidae) and form the sole hosts of the three genera of the subfamily LERNENTOMINAE of the CHONDRACANTHIDAE. This subfamily is the most degenerate of the family, having in addition to the head a long cephalic neck buried in the tissues of the host, the segmentation is less distinct than in *Chondracanthus*, the thoracic appendages are non-articulate, and the antennules one or two articled (Leigh-Sharpe and Oakley, 1927). The three genera *Lernentoma*, *Oralien* and *Medesicaste* are all parasitic on *Trigla* spp.

(11) The BALISTIFORMES harbour very degenerate parasites: *Naobranchia*, an aberrant genus of the LERNAEOPODIDAE, of which various species occur on Diodontid and Tetradontid globe-fishes; *Naobranchia stibara* Leigh-Sharpe, 1926, occurs on *Chilomycterus spinosus*.

(12) The ACANTHOPTERYGII, a vast group, harbour the CHONDRACANTHIDAE, which are not deeply imbedded, the PHILICHTHYIDAE, the DICHELESTHIIDAE (but several of the genera of this family are found on Elasmobranchs) a family in which the head is small, body elongated, thoracic segments free and simple, and the antennules have rarely as few as 2 or 3 articles (8 in *Dichelesthium*, 7 in *Krøyeria*, 6 in *Congericola*, 5 in *Eudactylina*, *Hatschekia*, etc.), and the CALIGIDAE, which can leave their hosts if necessary (*e.g.* on the death of the host), and differ but little from free-living forms (antennules however 2–3 articled).

(13) In the ZEORHOMBIFORMES the flat-fishes are regarded as comparatively recent, and on their fin-rays are found such visiting parasites as the CALIGIDAE and the bulk of the ERGASILIDAE, especially the genera *Caligus* and *Lepeophtheirus* in the first family and *Bomolochus* in the second, *e.g. C. zei* on *Zeus faber* (*vide* Oakley, 1926), *L. pectoralis* on all kinds of flat-fish, *B. soleae* on *Solea*, *B. zeugopteri* on *Zeugopterus*. The Ergasilidae are Cyclopoid and have as many as 5–7 articles in the antennule.

(14) The hosts of the SPHYRIIDAE are for the most part unknown or of a varied nature, *e.g. Genypterus blacodes*, *Anarrhichas lupus*, *Cyclopterus*

lumpus, *Macrourus*, *Synaphobranchus*, etc. The family is most degenerate and two genera at least, *Paeon* and *Tripaphylum*, are found on Elasmobranchs.

(15) In marine MAMMALIA two species of *Pennella* of the family Lernaeidae have succeeded in establishing themselves on Cetaceans; *P. balaenopterae* on *Balaenoptera musculus*, and *P. crassicornis* on *Hyperoodon rostratus*; thus a moderately ancient group of mammals bears a moderately degenerate Copepod.

(16) The MONSTRILLIDAE, a family of Copepods free-living when adult, are nevertheless peculiar in not possessing several of the usual pairs of swimming appendages; correlated with this is the fact that some genera, *e.g. Haemochera danae*, possess larval forms parasitic on Terebellid worms. Unless this highly degenerate larva is secondary, it points to this family being an ancient one.

(17) It should be noted that the genus *Pennella*, parasitic on Mammals, the highest of Vertebrates, has larval forms parasitic on CEPHALOPODA, the most highly evolved MOLLUSCA.

(18) The fact that the LICHOMOLGIDAE, resembling free-swimming Cyclopidae, are commensal in the mantle cavities of LAMELLIBRANCHIATA, seems to have no bearing on the present argument.

(19) The species of the most degenerate families, *e.g.* the LERNAEOPODIDAE, are constant to one species in their choice of a host, while those which are able to change from one host to another, especially the commoner species of Caligidae (*e.g. Caligus rapax* and *Lepeophtheirus*) are catholic in their choice of hosts. This is another argument in favour of the view that this family has only recently taken to parasitism.

It thus seems to me to be proved that the extremely degenerate families are on the whole parasitic on Invertebrates, which appeared earliest in geological time, the very degenerate upon the earliest fishes, the degenerate upon later fishes, the barely degenerate upon the latest fishes and Ascidians; or, in other words there is a definite correspondence between the degree of degeneracy exhibited by the parasite and the distribution of its host in time.

Families of Parasitic Copepoda in order of their degeneracy.		Type of Host most favoured.
Group I. Commensal and resembling free-living forms, appendages and segmentation complete. More than seven articles in the antennule.		
	Ascidicolidae	Urochorda
	Notodelphyidae	Urochorda
	Lichomolgidae	Lamellibranchiata
Group II. Parasitic.	Antennules 11 articled	
	Ascomyzontidae (*Nicothoe*)	Crustacea Dekapoda
	Antennules 5–7 articled	
	Ergasilidae	Zeorhombiformes
	Antennules few-jointed, loss of segmentation	
	Choniostomatidae	Higher Crustacea
	Antennules 2–3 jointed	
	Caligidae	Acanthopterygii

Families of Parasitic Copepoda in order of their degeneracy.	Type of Host most favoured.
Group III. Fixed parasites, partial loss of segmentation and appendages	
Dichelesthiidae	Acanthopterygii
Philichthyidae	Acanthopterygii
Chondracanthidae	Acanthopterygii
Lernentominae	Trigliformes
Lernaeidae	Physostomi Gadiformes Crossopterygii
Lernaeopodidae	Gadiformes Salmoniclupeiformes Elasmobranchii
Sphyriidae	?
Group IV. Burrowing parasites, loss of segmentation, appendages and alimentary canal	
Tanypleurus	*Somniosus*
Herpyllobiidae	Polynoidae
Saccopsidae	Terebellidae

SUMMARY.

Phylum, etc. of Host			Parasite
ANNULATA	POLYCHAETA	Nereidae	Nereicolidae
		Terebellidae	Saccopsidae
		Polynoidae	Herpyllobiidae
ECHINODERMATA	OPHIUROIDEA		*Arthrochordeumium*
			Cancerillidae
ARTHROPODA	CRUSTACEA		*Nicothoë*
			Choniostomatidae
CHORDATA	UROCHORDA		Ascidicolidae
			Notodelphyidae
	VERTEBRATA	PISCES	
		Elasmobranchii	*Tanypleurus*
			Lernaeopodidae
		Crossopterygii	*Lernaea haplocephala*
		Holocephali	*Chondracanthus epachthes*
		Teleostei	
		Physostomi	Lernaeopodidae
			Lernaeidae
		Anacanthini (Gadiformes)	Lernaeopodidae
		Trigliformes	Lernaeopodidae
			Lernentominae
		Acanthopterygii	Chondracanthidae
			Philichthyidae
			Dichelesthiidae
			Caligidae
		Zeorhombiformes	Caligidae
			Ergasilidae
		MAMMALIA	
		Cetacea	*Pennella*

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IMMUNOLOGICAL STUDIES OF *TRYPANOSOMA GAMBIENSE* AND *T. RHODESIENSE*.

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Introduction.

During the eighteen months spent with the League of Nations International Commission on Human Trypanosomiasis, I had a unique opportunity for studying the transmissibility of the human trypanosomes by *Glossina palpalis*.

In the course of these studies it became evident that this character possessed great interest for the epidemiologist. A number of the human strains that passed through my hands at the Entebbe Laboratory was found, either in man himself or on first isolation in laboratory animals, to be non-transmissible by the cyclical method by tsetse. Other strains, though readily transmissible when first isolated, were found to lose this property after a longer or shorter sojourn in the mammal, whether examined in monkeys or in one of the domesticated ruminants.

These observations, recorded in the Final Report of the Commission (Duke, 1928), suggested an explanation of some of the many puzzling features presented by the incidence and spread of human trypanosomiasis. In Paper No. 5 of my contribution to this Report, I attempted to apply the results obtained in the laboratory to the solution of these problems. The evidence supplied by numerous experiments suggested that it is a general rule for the trypanosomes of the polymorphic group to lose their transmissibility by tsetse after more or less prolonged sojourn in the blood of a mammal. The animals used by the Commission were monkeys and domesticated ruminants. Experiments with antelope were planned, but were, of necessity, postponed. There is some reason to believe that the polymorphic trypanosomes attain a higher level of biological adjustment in certain species of African antelope than in any other host.

After a brief review of the available evidence, the conclusion was reached, provisionally, that it is of supreme importance to *Trypanosoma gambiense* (and probably also to other species of trypanosome) that the vertebrate host be frequently exposed to tsetse during the early stages of its infection, before the transmissibility of the strain is impaired.

In nature this requisite will be fulfilled when susceptible hosts are numerous and the contact between them and the fly is close. Then the trypanosome will be found widely distributed, *i.e.* a high percentage of the principal vertebrate hosts will be infected, and the "transmissibility index" of the strains will be high. Furthermore, it was pointed out that this frequent passage of the parasite from host to host might aid the propagation of the trypanosome in another way, by affording frequent opportunity *for reinfection or superinfection of the same host by fresh strains.*

Let us now consider the work done up to date on this particular line of investigation.

The main objects of the experiments we are about to consider were:

1. To determine whether infection of a mammal with *T. gambiense* confers any immunity against subsequent infection by (*a*) the same strain, (*b*) a different strain of this trypanosome, or (*c*) by *T. rhodesiense* or *T. brucei.*

2. If infection results in the above circumstances and no solid immunity is demonstrated, we then have to inquire whether there is any finer or less obvious reaction produced by a long-standing infection: such, for example, as might be manifested by changes in the transmissibility by tsetse of succeeding infecting strains of trypanosomes, when these are introduced into the blood stream of the animal under investigation.

To investigate this, an infected animal was selected in which the strain of trypanosomes had, in the course of time, lost its transmissibility. Flies, infective with a readily transmissible strain of *T. gambiense*, from the same or a different patient, were then fed upon the animal. After a sufficient incubation period had elapsed for the new strain to appear in the peripheral circulation, clean flies were applied and the transmissibility of the now bivalent infection examined. In some instances the investigation was extended to include a third exposure to infection, after a suitable interval.

Concurrently, similar experiments were performed in which animals carrying a strain of *T. gambiense* that had become non-transmissible by tsetse were superinfected by flies carrying either *T. rhodesiense* or the Damba trypanosome (*T. brucei* type).

In all these experiments the technique described in the Final Report of the Commission was employed. The experimental animals—monkeys, sheep, and goats—were all kept under close observation, and guarded against casual infection. Each individual animal was examined daily, some Sundays excepted, by the stained thick-film method, and never has any instance of accidental infection been detected. Controls were employed freely. The results, therefore, are above suspicion on the score of contaminating infections.

The method of calculating the transmissibility index is given in full in the Final Report. For the sake of clearness the formula for calculating this index is reproduced:

$$T = \frac{s}{p} \times \frac{n \times 100}{N},$$

where N = total number of flies dissected; n = total number of flagellate-containing flies; p = number of infected flies of twenty-five days old or more; and s = the number of these mature infected flies that shows salivary gland infection. The fraction $\frac{s}{p}$ = *metacyclic index* of the strain. The percentage of all flies dissected during a "test" that contains flagellates is called the *infectivity index* of the strain.

Before proceeding to the actual experiments, a word of warning is, perhaps, expedient. The experiments under review, as well as those presented here for the first time, have one defect in common. The number of flies employed is, of necessity, small, and, mathematically, there is a wide margin of error in drawing inferences from data of this kind.

Efforts have been and are being made, by concentrating attention on one or two strains, to remedy this shortcoming. Direct confirmation of the views I have put forward here and in the Report of the Commission is difficult to secure. But if the interpretation of the results of the transmission experiments of the last two years is wrong, then, sooner or later, the investigations still in progress are bound to reveal the fact; at the same time, in the absence of such disproof, every additional experiment diminishes the likelihood of error.

In view of the long duration of experimental work of this kind I think it best to set forth, from time to time, the progress—or apparent progress—made, so that no opportunity may be lost by other workers to join in the search for the truth.

The results of the experiments now to be considered are given in two kinds of tables. One table, headed *Transmission Experiments*, deals with the actual transmission experiments. The other, given first in each case, presents the essential data supplied by these experiments abridged and arranged to facilitate comparison with other strains.

Each set of transmission experiments is thus represented in the corresponding *transmissibility index table* by a summary, A, B, C, etc., under the same date. In the transmissibility index table there is often included, for clearness sake, the summary of experiments with the same animal already published in the Report (Duke, 1928).

In the column headed "Ages of the infected flies" the figures between semi-colons relate to flies with salivary gland infections.

I. Experiments performed.

We will now take in turn the animals tested, giving in each case the history of all manipulations to which each was subjected.

In what follows an "infective fly" is a fly whose salivary glands are infected with the metacyclic forms of the strain of trypanosome under consideration. Such a fly infects every susceptible animal that it bites.

Monkey 95.

This animal was infected on 23. v. 26 by two infective flies. These flies derived their infection from Monkey 64, which, in turn, had been sub-inoculated with the blood of a patient from South Kavirondo on 16. iii. 26.

This strain is *T. gambiense* Strain No. V of the Commission's Reports. The history of the strains studied by the Commission is given in full in the Final Report.

The examinations previously made of the transmissibility of this strain in Monkey 95 (cf. Final Report) are recorded in Tables I and II.

Table I. *Transmissibility Index* (*Monkey* 95).

Date	Total flies dissected	Total infected flies	Percentage of infected flies	Number of infected flies alive on 25th day: With glands infected	Number of infected flies alive on 25th day: Total	Percentage of gland infections	Transmissibility index
			A.				
x. 26	89	15	16·8	2	5	2·2	**6·7**
ii. 27	77	3	3·8	3	3	3·8	**3·8**
v and vi. 27	228	5	2·1	0	4	0	**0**
			B.				
vii and viii. 27	376	7	1·8	3	5	0·7	**1·0**
viii and ix. 27	324	0	0	0	0	0	**0**
xi and xii. 27	181	4	2·2	0	1	0	**0**
			C.				
xii. 27 and i. 28	335	14	4·1	7	11	2·0	**2·6**

Section A of Table I suggests that the strain was undergoing a progressive decrease in transmissibility. The last examination, in May and June, 1927, was significant both in respect of the number of flies used and the fact that four of the positive flies lived more than 32 days.

Table II. *Transmission Experiments* (*Monkey* 95).

Exp. No.	Day of experiment on which dissection began	Number of flies alive on 25th day	Number dissected during the experiment: Males	Females	Total	Duration of experiment in days	Number of flies containing flagellates: In gut only	In gut and salivary glands	Ages of the infected flies dissected during experiment	Dates of infecting feeds
						A.				
578	8	32	24	24	48	31	1	0	31	25, 27, 28. vii. 27
583	11	18	19	23	42	34	1	0	25	27, 29, 30. vii. 27
587	11	29	31	14	45	33	1	0	16	28, 30, 31. vii. 27
592	9	30	39	25	64	32	1	1	11 ; 29 ;	29, 30. vii & 1. viii. 27
599	12	32	24	23	47	33	0	2	; 33 ; 33 ;	31. vii & 2, 3. viii. 27
602	8	25	25	16	41	36	0	0	—	1, 3, 4. viii. 27
605	11	24	25	21	46	34	0	0	—	2, 4, 5. viii. 27
608	8	29	23	20	43	32	0	0	—	4, 6, 7. viii. 27
						B.				
632	9	32	15	23	38	31	0	0	—	29, 31. viii & 1. ix. 27
637	12	25	22	23	45	33	0	0	—	1, 3, 4. ix. 27
638	6	22	14	28	42	32	0	0	—	2, 4, 5. ix. 27
643	6	19	20	19	39	31	0	0	—	5, 7, 8. ix. 27
646	7	19	20	24	44	31	0	0	—	6, 8, 9. ix. 27
656	9	33	28	15	43	32	0	0	—	9, 11, 12. ix. 27
658	11	26	12	20	32	31	0	0	—	10, 12, 13. ix. 27
662	8	25	16	25	41	31	0	0	—	11, 13, 14. ix. 27
						C.				
761	8	11	18	24	42	32	2	0	15, 15	19, 21, 22. xi. 27
765	6	—	15	11	26	23	0	0	eaten by ants	21, 23, 24. xi. 27
774	7	—	10	7	17	18	0	0	eaten by ants	25, 27, 28. xi. 27
776	6	15	18	7	25	32	2	0	9, 31; only 2 tryps. seen	26, 28, 29. xi. 27
778	7	30	29	7	36	30	0	0	—	29, 30. xi & 1. xii. 27
784	7	21	26	9	35	31	0	0	—	2, 4, 5. xii. 27
						D.				
817	8	8	20	14	34	26	1	0	10	28, 30, 31. xii. 27
819	10	23	27	11	38	25	1	2	23 ; 23 ; 23 ;	30. xii. 27; 1, 2. i. 28
824	11	19	11	16	27	25	1	2	25 ; 22 ; 20 ;	1, 3, 4. i. 28
827	7	23	18	22	40	25	2	0	20, 20	2, 4, 5. i. 28
830	7	13	12	13	25	28	0	0	—	7, 8, 10. i. 28
835	9	24	28	20	48	28	0	2	; 28 ; 28 ;	7, 9, 10. i. 28
847	7	20	12	26	38	28	0	0	—	13, 15, 16. i. 28
852	6	14	26	11	37	28	1	0	14	16, 18, 19. i. 28
854	7	17	27	21	48	27	1	1	18 ; 27 ;	17, 19, 20. i. 28

Note. Section B of this table illustrates the need for care imposed by the limitations attending this method of examination of the transmissibility of a strain. An entirely negative set of experiments is followed a month later by evidence that the strain can still develop in the fly's gut. The single fly in section C that lived more than 25 days had, however, an exceedingly slight infection of the intestine, only two or three flagellates being found in the whole preparation. This phenomenon, in my experience, is itself an indication of impairment of the transmissibility of the strain of tsetse.

Superinfection of Monkey 95.

(*a*) *With a homologous strain.* On 13 and 14. vii. 27 the monkey was fed on by a fly infective with Strain V, derived from Monkey 358 which represented the eighth passage from the original patient (transmissibility index of the strain in Monkey 358 was **1·3**). Five of these passages had been effected by cyclically infected tsetse; three (the first two and the last) by the syringe. After this superinfection, the examinations recorded in Table I, section B, and Table II, sections A, B, and C, were made. Full details are given as these experiments are hitherto unpublished.

Apparently, the superinfection of this monkey with a later generation of the strain that originally infected it, produced a temporary recrudescence of the transmissibility of the trypanosomes in the animal's blood.

Presumably the index of **1·0** in Monkey 95 (Table I, section B) related to the newly introduced strain. The index, however, rapidly fell again to zero, indicating that some kind of resistance had been established in the monkey as a result of its prolonged struggle with Strain V.

The possession by this monkey of some kind of resistance was also manifested clinically by the general state of the animal, which remained full of life and vigour and to all appearances in excellent health. All this time trypanosomes were demonstrable in its peripheral blood. No examination of the cerebro-spinal-fluid of this monkey was made. The operation of puncturing the membranes at the base of the skull is a difficult one, especially with a small animal like No. 95, whose value in many respects is unique.

(*b*) *With a heterologous strain.* On 17 and 19. xii. 27 the monkey was fed on each day by one of two flies, both infective with another strain (Ibrahim) of *T. gambiense*. The transmissibility index of this strain was **3·9**. These flies had obtained their infection by feeding on Ibrahim himself. The transmission experiments performed after this superinfection are shown in Table II, section D, and Table I, section C.

Once again, superinfection—this time with a different strain from that to which the monkey had become accustomed—was followed by a recrudescence of transmissibility.

This time the index is **2·6**, and it is highly probable that this is ascribable to the new strain circulating in the monkey's blood. Four months after the second superinfection the monkey still appeared to be in good condition, but sufficient time has not yet elapsed to pronounce definitely on this point. Nor has it been possible to determine whether this latest accession of transmissibility will disappear again as rapidly as did the first restoration. Experiments carried out in April, 1928, demonstrated an *infectivity* index of 1·6, and from the incomplete data available at the time of writing it is evident that the transmissibility index was also considerably reduced. The strain was, however, still transmissible at that date.

Exp. No.

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834
840
841
851

Sheep 333.

A large, powerful and vigorous ram. Originally infected on 20. iv. 26, by the syringe, from Monkey 64, which, in its turn, had been inoculated with *T. gambiense* direct from man.

Table III. *Transmissibility Index* (*Sheep* 333).

Date	Total flies dissected	Total infected flies	Percentage of infected flies	Number of infected flies alive on 25th day: With glands infected	Number of infected flies alive on 25th day: Total	Percentage of gland infections	Transmissibility index
			A.				
vii. 26	150	9	6·0	5	7	3·3	4·2
i–ii. 26	252	2	0·7	0	2	0	0
			B.				
vii–viii. 27	300	0	0	0	0	0	0
			C.				
viii–ix. 27	553	3	0·5	0	3	0	0
			D.				
xii. 27–i. 28	290	0	0	0	0	0	0

Table IV. *Transmission Experiments* (*Sheep* 333).

Exp. No.	Day of experiment on which dissection began	Number of flies alive on 25th day	Number dissected during the experiment: Males	Females	Total	Duration of experiment in days	Number of flies containing flagellates: In gut only	In gut and salivary glands	Ages of the infected flies dissected during experiment	Dates of infecting feeds
					A.					
572	9	38	29	18	47	31	0	0	—	22, 24, 25. vii. 27
576	9	26	31	20	51	30	0	0	—	24, 26, 27. vii. 27
594	14	33	16	24	40	33	0	0	—	29, 31. vii & 1. viii. 27
597	8	32	16	27	43	34	0	0	—	30. vii & 1, 2. viii. 27
598	9	25	14	15	29	34	0	0	—	31. vii & 1, 3. viii. 27
600	6	28	32	16	48	32	0	0	—	4, 6, 7. viii. 27
610	9	31	20	22	42	32	0	0	—	5, 7, 8. viii. 27
					B.					
634	7	32	24	24	48	31	1	0	31	30. viii & 1, 2. ix. 27
636	13	42	24	27	51	31	0	0	—	31. viii & 2, 3. ix. 27
642	4	24	22	21	43	31	2	0	31, 31	4, 6, 7. ix. 27
644	6	18	15	12	27	31	0	0	—	5, 7, 8. ix. 27
651	7	35	18	23	41	31	0	0	—	8, 10, 11. ix. 27
654	9	19	19	11	30	32	0	0	—	9, 11, 12. ix. 27
659	9	19	8	17	25	31	0	0	—	10, 12, 13. ix. 27
668	9	25	14	24	38	31	0	0	—	13, 15, 16. ix. 27
670	8	29	21	16	37	31	0	0	—	14, 16, 17. ix. 27
673	5	20	17	16	33	31	0	0	—	15, 17, 18. ix. 27
680	8	29	20	22	42	30	0	0	—	19, 21, 22. ix. 27
682	8	26	27	11	38	31	0	0	—	20, 22, 23. ix. 27
687	4	22	18	25	43	29	0	0	—	23, 25, 27. ix. 27
689	7	23	20	17	37	30	0	0	—	24, 26, 27. ix. 27
691	7	8	13	7	20	30	0	0	—	25, 26, 28. ix. 27
					C.					
814	7	13	21	11	32	29	0	0	—	27, 29, 30. xii. 27
816	7	16	21	20	41	29	0	0	—	28, 30, 31. xii. 27
820	10	17	16	7	23	31	0	0	—	30. xii. 27; 1, 2. i. 28
829	7	12	21	19	40	28	0	0	—	3, 5, 6. i. 28
831	7	6	10	8	18	28	0	0	—	4, 6, 7. i. 28
834	7	17	24	16	40	28	0	0	—	6, 7, 9. i. 28
840	7	29	29	13	42	28	0	0	—	10, 12, 13. i. 28
841	7	13	15	21	36	28	0	0	—	11, 13, 14. i. 28
851	10	11	17	1	18	29	0	0	—	15, 17, 18. i. 28

The experiments summarised in Table III, section A, were described in the Report of the Commission (Duke, 1928). Two months and a half after its original infection the strain in this sheep was readily transmissible. Six months later the infectivity was much reduced and the transmissibility index zero. One more test (Table III, section B, and Table IV, section A) was made before superinfection. Apparently the strain had lost all power to develop in tsetse, the animal having completely "recovered" from its infection.

Superinfection.

(*a*) *With a homologous strain* (Table III, section C, and Table IV, section B).

On 19 and 20. viii. 27 one infective fly fed on this sheep.
On 20 and 21. viii. 27 another infective fly fed on this sheep.
On 25, 26 and 28. viii. 27 two more infective flies fed on this sheep.
On 30. viii. 27 another infective fly fed on this sheep.

The first two of these flies carried trypanosomes derived from the eighth passage of Strain V from man; the other three flies, trypanosomes from the ninth passage.

This series is fairly convincing, all the positive flies living for at least 31 days. It suggests that a transitory re-establishment of trypanosomes occurred in the sheep after these five flies had fed upon it, but the resulting infection was not transmissible. We see how delicate is this transmissibility test in detecting fine degrees of immunity. A *nuance* is here revealed that without this test would have passed unsuspected.

(*b*) *With a heterologous strain* (Table III, section D, and Table IV, section C). On 15 and 21. xii. 27 one infected fly carrying a different human strain (Ibrahim) fed on the sheep.

This attempt at reinfection with another strain of *T. gambiense* failed. Apparently this animal had acquired an absolute immunity from *T. gambiense*. It is still being examined, both by the fly-test and by blood slide. For months past, the routine examination of its peripheral blood has failed to reveal any trypanosomes.

The next two sets of experiments, with Monkey 375 and Sheep 161, are less instructive, because at the time of superinfection the strain was still transmissible.

Monkey 375.

Originally infected on 3. vii. 27, by a fly carrying Strain V. This fly derived its infection from the seventh passage of the strain from man.

Exp. No.
563
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762
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772
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823
825
830
839
845

Table V. *Transmissibility Index* (*Monkey* 375).

Date	Total flies dissected	Total infected flies	Percentage of infected flies	Number of infected flies alive on 25th day: With glands infected	Number of infected flies alive on 25th day: Total	Percentage of gland infections	Transmissibility index
			A.				
vii. 27	253	8	3·1	3	6	1·1	1·5
xi. 27	106	1	0·5	1	1	0·5	0·5
			B.				
xii. 27–i. 28	268	11	4·1	4	10	1·4	1·6

Table VI. *Transmission Experiments* (*Monkey* 375).

Exp. No.	Day of experiment on which dissection began	Number of flies alive on 25th day	Number dissected during the experiment: Males	Females	Total	Duration of experiment in days	Number of flies containing flagellates: In gut only	In gut and salivary glands	Ages of the infected flies dissected during experiment	Dates of infecting feeds
					A.					
563	9	32	23	18	41	30	0	0	—	18, 20, 21. vii. 27
566	10	33	20	25	45	38	1	0	12	19, 21, 22. vii. 27
570	7	30	19	20	39	36	0	1	; 36 ;	21, 22, 24. vii. 27
575	10	24	15	20	35	37	1	1	25 ; 37 ;	23, 25, 26. vii. 27
577	9	38	20	27	47	36	0	0	—	24, 26, 27. vii. 27
586	11	39	19	27	46	30	3	1	11, 30, 30 ; 22 ;	
					B.					
758	6	22	22	12	34	32	0	0	—	18, 20, 21. xi. 27
759	7	6	17	19	36	31	0	0	—	19, 21, 22. xi. 27
762	6	11	31	15	46	33	0	0	—	20, 22, 23. xi. 27
769	7	19	22	17	39	34	0	0	—	23, 25, 26. xi. 27
772	7	25	15	16	31	33	0	1	; 33 ;	24, 26, 27. xi. 27
					C.					
812	8	27	20	15	35	30	1	1	26 ; 30 ;	24, 26, 27. xii. 27
813	7	8	12	8	20	28	0	0	—	26, 28, 29. xii. 27
815	7	13	20	10	30	28	0	0	—	27, 29, 30. xii. 27
823	7	21	19	14	33	25	0	1	; 17 ;	1, 3, 4. i. 28
825	8	16	14	12	26	28	1	0	28	2, 3, 5. i. 28
830	8	29	24	14	38	28	1	1	28 ; 28 ;	4, 6, 7. i. 28
839	7	17	17	21	38	28	2	0	11, 28	10, 12, 13. i. 28
845	8	19	27	21	48	29	2	1	17, 19 ; 29 ;	11, 13, 14. i. 28

Some four and a half months after the original infection of Monkey 375 the strain was still transmissible (Table V, section A, and Table VI, section B).

Superinfection.

On 11. xii. 27, the monkey was inoculated with the infected glands of a fly carrying another strain of *T. gambiense* (Ibrahim); and on 13. xii. 27 it was fed on by a fly infective with this strain.

The index of Strain Ibrahim was **3·9**. There is nothing very much to learn from this experiment. The strain in Monkey 375 was still transmissible when

superinfection occurred. Possibly a revival of transmissibility took place after the introduction of Strain Ibrahim. This monkey, like 95, appeared to be in good condition at the time when these experiments were carried out. The animal died from trypanosomiasis on 20. v. 28.

Table VI, section B, and, still more, Table XII, section A, call for a word of explanation. The reasons that have led to the advocacy of this method of studying the transmissibility of trypanosomes are stated in the Report already referred to, in Paper No. 5; they cannot all be repeated here. The method depends on the assumption that the varying factor in the combine is the trypanosome, not the tsetse. It is known that, in any series of xenodiagnostic experiments with tsetses and their trypanosomes, the number of infected flies varies considerably according to the dates of their feeds on the infecting animal. A box of flies fed on Monday may show 10 per cent. of infected flies, another applied on Tuesday, nil; and so on.

The technique here recommended aims at applying flies to the infected animal during one complete revolution of what Robertson has called the endogenous cycle (Robertson, 1913). Little is known about the periodicity of this cycle or the limits within which it may vary.

Evidently, then, it is possible for a "test" to coincide with a negative phase of the cycle, and caution is therefore needed before a strain is pronounced non-transmissible.

At the present time my own opinion is that at the outset of an infection of an animal with a transmissible strain, the endogenous cycles are of short duration, say 7–10 days, so that, provided a reasonable number of flies has access to the animal each day for a week or so, the results are fairly reliable. Naturally, the more flies applied the better the test.

Exp. No.

753
754
763
764
767

On the other hand, I think there is evidence to show that in long-standing yet still transmissible infections, the revolutions of the endogenous cycle become slower, so that in making a test a longer period must be covered.

809
810
811
818
826
828
838
846

And, finally—and here we step still further into the realm of speculation—I believe that in animals where a high degree of adjustment has been reached between host and parasite, a condition obtains where the *infectivity index* is very low but where the forms of the parasite that infect the fly are *relatively* numerous. When this stage is reached a large number of flies is needed to reveal an infection, but the *metacyclic index* of the strain (cp. p. 429) will approximate unity.

Sheep 161.

Originally infected by a fly on 24. viii. 26 from Sheep 333.

The earlier published experiments with this sheep may be summarised as follows:

Table VII. *Transmissibility Index* (*Sheep* 161).

Date	Total flies dissected	Total infected flies	Percentage of infected flies	Number of infected flies alive on 25th day: With glands infected	Number of infected flies alive on 25th day: Total	Percentage of gland infections	Transmissibility index
			A.				
x. 26	252	27	10·1	12	22	5·1	5·5
i and ii. 27	224	3	1·3	0	1	0	0
			B.				
xi. 27	192	3	1·4	1	3	0·4	0·4
			C.				
xii. 27–i. 28	236	4	1·6	0	1	0	0

Section A indicates a striking drop in both the infectivity and the transmissibility of the strain. In the second set of experiments, however, only one of the three positive flies lived until the 25th day. The next series of experiments performed in November, 1927, afforded a surprise (Table VII, section B, Table VIII, section A).

Table VIII. *Transmission Experiments* (*Sheep* 161).

Exp. No.	Day of experiment on which dissection began	Number of flies alive on 25th day	Number dissected during the experiment: Males	Females	Total	Duration of experiment in days	Number of flies containing flagellates: In gut only	In gut and salivary glands	Ages of the infected flies dissected during experiment	Dates of infecting feeds
					A.					
753	7	10	25	18	43	30	0	0	—	15, 17, 18. xi. 27
754	6	34	28	17	45	33	0	0	—	16, 18, 19. xi. 27
763	8	16	26	10	36	33	1	0	33	20, 22, 23. xi. 27
764	8	20	19	18	37	33	0	1	; 33 ;	21, 23, 24. xi. 27
767	11	19	20	11	31	32	1	0	32	22, 24, 25. xi. 27
					B.					
809	11	12	13	6	19	32	0	0	—	22, 24, 25. xii. 27
810	9	8	14	16	30	31	1	0	10	24, 26, 27. xii. 27
811	9	10	8	13	21	31	0	0	—	24, 26, 27. xii. 27
818	9	15	20	9	29	29	2	0	18, 19	29, 31. xii & 1. i. 28
826	7	13	22	8	30	29	0	0	—	2, 4, 5. i. 28
828	12	24	23	12	35	28	1	0	28	3, 5, 7. i. 28
838	9	23	24	20	44	28	0	0	—	9, 11, 12. i. 28
846	9	18	15	13	28	28	0	0	—	13, 14, 16. i. 28

In this sheep the trypanosome strain retained its transmissibility for no less than 15 months, and this suggests that in certain circumstances sheep may act as a reservoir for *T. gambiense*.

Superinfection.

(Table VII, section C, and Table VIII, section B.)

On 11. xii. 27 the sheep was fed on by a fly infective with Strain Ibrahim. Unfortunately in this series only one of the four positive flies live 25 days, so that the series is inconclusive. The infectivity index (cp. p. 429) shows no material alteration.

Sheep 332.

This animal was infected with a strain of *T. gambiense* (No. II of the Commission's Report) possessed of a low degree of transmissibility. Only one infective fly was ever obtained in all those applied to different animals infected with this strain.

Sheep 332 was infected by the syringe on 31. iii. 26 from Monkey 53, which had been inoculated with the patient's blood. The earlier experiments with this sheep gave the following results (Table IX, section A).

Table IX. *Transmissibility Index* (*Sheep* 332).

Date	Total flies dissected	Total infected flies	Percentage of infected flies	Number of infected flies alive on 25th day: With glands infected	Number of infected flies alive on 25th day: Total	Percentage of gland infections	Transmissibility index
			A.				
v. 26	70	3	4·2	0	2	0	0
vii–viii. 26	192	3	1·5	0	3	0	0
iv. 27	223	4	1·7	0	4	0	0
			B.				
ix. 27	422	1	0·2	0	1	0	0
			C.				
xi. 27	256	15	5·8	5	9	1·9	3·2

The strain in this sheep was thus apparently non-transmissible (cp. section A). In the last set of the section all four infected flies lived 25 days or more.

Table X. *Transmission Experiments* (*Sheep* 332).

Exp. No.	Day of experiment on which dissection began	Number of flies alive on 25th day	Number dissected during the experiment: Males	Females	Total	Duration of experiment in days	Number of flies containing flagellates: In gut only	In gut and salivary glands	Ages of the infected flies dissected during experiment	Dates of infecting feeds
					A.					
650	8	30	27	10	37	31	1	0	31	7, 9, 10. ix. 27
653	7	26	26	11	37	31	0	0	—	8, 10, 11. ix. 27
655	7	24	19	17	36	32	0	0	—	9, 11, 12. ix. 27
666	12	32	21	22	43	31	0	0	—	12, 14, 15. ix. 27
669	8	18	21	13	34	31	0	0	—	13, 15, 16. ix. 27
671	7	33	25	16	41	31	0	0	—	14, 16, 17. ix. 27
677	8	26	16	22	38	31	0	0	—	17, 19, 20. ix. 27
679	9	33	24	23	47	31	0	0	—	18, 20, 21. ix. 27
686	10	27	21	20	41	30	0	0	—	22, 24, 25. ix. 27
688	5	23	17	21	38	30	0	0	—	24, 26, 27. ix. 27
692	8	17	18	12	30	30	0	0	—	25, 26, 28. ix. 27
					B.					
771	8	19	17	12	29	33	1	0	33	24, 26, 27. xi. 27
773	8	21	22	11	33	34	1	0	34	25, 27, 28. xi. 27
775	6	20	28	16	44	32	3	1	8, 11, 32 ; 32 ;	26, 28, 29. xi. 27
780	5	15	12	17	29	30	0	1	; 30 ;	29. xi & 1, 2. xii. 27
783	8	21	17	14	31	30	2	0	8, 25	30. xi & 1, 2. xii. 27
786	5	20	20	9	29	33	1	2	14 ; 30 ; 33 ;	3, 5, 6. xii. 27
788	8	16	15	18	33	30	2	0	17, 18	4, 6, 7. xii. 27
792	5	18	21	7	28	28	0	1	; 26 ;	5, 7, 8. xii. 27

Table X shows experiments performed after superinfection; section A after *T. gambiense*, section B after *T. rhodesiense* (cp. also Table IX, sections B and C respectively).

Superinfection.

(*a*) With *T. gambiense* (Strain V). On 25. viii. 27, one infective fly; on 26 and 27. viii. 27, one infective fly; and on 1. ix. 27, two infective flies fed on the sheep. All these flies had been infected cyclically from Monkey 375.

A large number of flies was used in these experiments, and it is evident that neither the transmissibility index nor the total percentage of positive flies had increased as a result of the superinfection.

Believing the absence of salivary gland infections from the flies of the experiments of Table IX, section A, to be due to the Strain No. II having already become non-transmissible before the sheep was first infected, I expected a positive result after superinfection with a transmissible strain of *T. gambiense*. Apparently, however, this animal was able to exert an inhibitive influence on Strain V, either preventing altogether the trypanosome from appearing in the blood, or modifying its attributes and making it non-transmissible.

Incidentally, this particular experiment is an example of what has already been recognized as occurring in nature, namely, a transmissible strain losing this property on being transferred by an infective fly into another host.

(*b*) With *T. rhodesiense*.

On 13 and 15. xi. 27 a single infective fly fed on this sheep.
On 15. xi. 27 another infective fly fed on this sheep.
On 17 and 18. xi. 27 two other infective flies fed on this sheep.
On 18. xi. 27 two other infective flies fed on this sheep.

All these flies were infective with the same strain of *T. rhodesiense*, its transmissibility index being **3·7**. Evidently the *T. rhodesiense* strain invaded Sheep 332 without let or hindrance, the indices in the monkey from which it came and in the sheep being almost identical.

There is thus a striking difference between the behaviour of the two trypanosomes in Sheep 332. Whereas the animal gave evidence of a definite resistance to *T. gambiense*—a resistance either inherent, or, more probably, conferred by exposure to long-standing infection with Strain II—it was readily susceptible to *T. rhodesiense*, which appeared unchanged in its blood after a normal incubation period, and eventually killed the animal. The sheep died on 12. i. 28: post mortem the spleen was enlarged, with a raspberry-jelly appearance on section, and death may be safely ascribed to *trypanosomiasis rhodesiensis*.

Goat 341.

Originally infected, on 12. v. 26, by inoculation by the syringe from Monkey 65 (Strain VI). The monkey had been inoculated direct from man. The earlier transmission experiments are shown in Table XI, section A.

Table XI. *Transmissibility Index* (*Goat* 341).

Date	Total flies dissected	Total infected flies	Percentage of infected flies	Number of infected flies alive on 25th day: With glands infected	Number of infected flies alive on 25th day: Total	Percentage of gland infections	Transmissibility index
			A.				
vii–viii. 26	235	6	2·5	0	5	0	0
vi. 27	200	1	0·5	0	1	0	0
viii. 27	304	1	0·3	0	1	0	0
			B.				
ix. 27	455	0	0	0	0	0	0
			C.				
xi. 27	191	12	6·2	5	9	2·6	3·4

Strain VI, from its first isolation, was always found to be non-transmissible. After 15 months in the goat the strain had nearly lost all power to develop in tsetse (Table XI, section A, and Table XII, section A).

Table XII. *Transmission Experiments* (*Goat* 341).

Exp. No.	Day of experiment on which dissection began	Number of flies alive on 25th day	Number dissected during the experiment: Males	Females	Total	Duration of experiment in days	Number of flies containing flagellates: In gut only	In gut and salivary glands	Ages of the infected flies dissected during experiment	Dates of infecting feeds
					A.					
620	9	23	28	29	57	32	0	0	—	16, 18, 19. viii. 27
621	10	36	28	18	46	31	0	0	—	17, 19, 20. viii. 27
624	11	25	17	18	35	31	0	0	—	20, 22, 23. viii. 27
625	9	16	16	8	24	31	0	0	—	22, 24, 25. viii. 27
628	8	19	27	17	44	31	0	0	—	23, 25, 26. viii. 27
629	9	23	14	20	34	30	0	0	—	24, 26, 27. viii. 27
630	9	22	12	22	34	31	0	0	—	27, 29, 30. viii. 27
633	13	23	16	14	30	31	1	0	31	29, 31. viii & 1. ix. 27
					B.					
645	10	30	19	21	40	30	0	0	—	5, 7, 8. ix. 27
647	9	28	25	16	41	31	0	0	—	6, 8, 9. ix. 27
649	14	21	8	15	23	31	0	0	—	7, 9, 10. ix. 27
657	9	33	28	15	43	32	0	0	—	9, 11, 12. ix. 27
661	15	33	20	25	45	31	0	0	—	11, 13, 14. ix. 27
665	6	35	19	22	41	31	0	0	—	12, 14, 15. ix. 27
674	10	24	22	18	40	31	0	0	—	15, 17, 18. ix. 27
675	9	29	17	24	41	32	0	0	—	16, 18, 19. ix. 27
678	10	13	24	12	36	31	0	0	—	17, 19, 20. ix. 27
684	6	20	18	21	39	30	0	0	—	21, 23, 24. ix. 27
685	10	18	15	19	34	30	0	0	—	22, 24, 25. ix. 27
690	6	10	19	13	32	30	0	0	—	25, 26, 28. ix. 27
					C.					
710	20	22	14	12	26	30	0	0	—	23, 24, 26. x. 27
713	6	25	17	25	42	35	0	0	—	24, 25, 27. x. 27
716	6	17	15	16	31	34	0	0	—	25, 26, 28. x. 27
722	6	13	24	10	34	33	0	0	—	28, 30. x. 27
727	6	23	23	16	39	32	1	1	26 ; 32 ;	31. x & 2. xi. 27
728	9	16	23	7	30	32	1	0	25	31. x & 2. xi. 27
734	7	26	15	25	40	32	4	1	11, 20, 24, 29 ; 32 ;	1, 2. xi. 27
736	10	32	19	22	41	32	1	2	32 ; 32 ; 32 ;	3, 4, 5. xi. 27
749	6	24	23	18	41	31	0	1	; 31 ;	13, 15, 16. xi. 27

Superinfection.

(*a*) *T. gambiense* (Table XI, section B, and Table XII, section B).

On 22 and 23. viii. 27 the goat was fed on by three infective flies.

On 30. viii. 27 it was fed on by two infective flies.

These flies carried *T. gambiense* (Strain V); the first three were infected from the eighth passage, the last two from the ninth passage of this strain from man.

Apparently this attempt to infect this goat with another transmissible strain of *T. gambiense* failed completely.

It is, of course, possible that trypanosomes appeared in the blood in a non-transmissible form. But a more probable explanation is failure to infect, due, presumably, to acquired resistance, amounting for all practical purposes to complete immunity.

(*b*) *With the Damba trypanosome.* (Table XI, section C, and Table XII, section C.) On 15 and 16. x. 27 the goat was fed on by an infective fly carrying the Damba trypanosome.

The index of this strain in the Monkey 387, whence the infecting fly derived its infection, was also **3·4**. The goat thus possessed no resistance against the Damba trypanosome, although the attempt to superinfect it with *T. gambiense* had failed altogether.

Section C of Table XII illustrates a point that has not yet been dealt with, namely, that the incubation period which elapses between the act of infection and the appearance of trypanosomes in the peripheral blood does not necessarily coincide with the time required for the appearance in the blood of those forms of the parasite that are concerned with infection of the tsetse. The first four experiments in the above series may be ignored in calculating the index, the flies to which they relate being put on the goat too soon after the introduction into its blood of the Damba Strain.

This and other similar observations indicate that the forms of the parasite suitable for development in the insect vector are not present in the blood, as a rule, during the first few days after trypanosomes first appear in the peripheral circulation.

II. DISCUSSION.

The behaviour of *T. gambiense* in the sheep and goats of these experiments is fairly consistent, and suggests that we are faced with genuine instances of immunity against a protozoan parasite.

The difficulty is to decide whether this immunity is natural, or acquired as the result of prolonged contact with the trypanosome.

With Sheep 333 the case seems clear. The animal contracted an infection with *T. gambiense*. For a time the strain was readily transmissible by tsetse. After some months this character disappeared, to be followed later on by all signs of infection; the animal had, in other words, completely recovered. At no time did the trypanosome cause any appreciable inconvenience to the sheep.

When, 16 months after the original infection, the animal was exposed to a descendant of the same strain of *T. gambiense*, the infection resulting was of a transitory nature, and the strain in the sheep non-transmissible. Four months later, another attempt to infect the sheep, this time with a different strain of *T. gambiense*, produced no recognisable effect.

There is no doubt that Sheep 333 possessed from the first a natural tolerance of *T. gambiense*, shown by the complete absence of appreciable symptoms. In the course of experimental manipulations it acquired, eventually, immunity against the trypanosome.

In addition, in the course of the development of immunity, we can perceive a preliminary phase, during which the trypanosome is still present in the blood but in a non-transmissible form. This phase probably always precedes, though it is not necessarily followed by the complete elimination of all traces of the trypanosome.

After the first attempt at superinfection, trypanosomes reappeared. But they were non-transmissible, and, at this stage, the sheep was non-infective to fly.

The response made by the tissues of the animal to the original infection, though insufficient completely to prevent superinfection, was sufficient to deprive the strain of its transmissibility.

Sheep 161 illustrates another type of reaction. In this animal, also, *T. gambiense* was readily established, causing no apparent symptoms. But here the trypanosome maintained its transmissibility for a period of at least 15 months. A few months after the original infection the transmissibility underwent a great reduction, and a condition was eventually reached resembling that already recognised in certain species of antelope, where the parasites, though very rare in the peripheral blood, are admirably adapted to transmission by tsetse. In both cases the infectivity index (*i.e.* total percentage of flies containing flagellates) and the transmissibility index are low.

The results obtained with Sheep 161 may necessitate some modification of the provisional conclusions I reached in the Final Report of the Commission, about the rôle of domestic ruminants in the spread of human trypanosomiasis. This is still under investigation. Unfortunately, we are once again confronted by the difficulty of being unable to experiment with man. The fact that a human strain can survive for 15 months in a sheep and retain its transmissibility for that length of time does not necessarily mean that the trypanosome, at the end of that period, is still capable of infecting man. But unless the pathogenicity for man is retained, we cannot regard the sheep as a reservoir of human trypanosomiasis.

The attempt to superinfect this sheep led to no definite conclusions. The absence of any striking increase in the infectivity index, suggests, however, that the animal, having once become adjusted to *T. gambiense*, no longer reacted to reinfection.

Sheep 332 also appears to have acquired immunity after prolonged contact

with *T. gambiense*. The strain in question was only feebly transmissible when first encountered, and never manifested this property in Sheep 332.

As the original inoculation into the sheep had been made by the syringe, it is possible, though highly improbable, that only non-transmissible forms of the trypanosome were included in the inoculum. On the other hand, the tissue-reactions of the sheep itself may have caused the transmissibility to disappear. There is no doubt that individual animals vary considerably in their susceptibility to *T. gambiense*. For example, two large rams employed by the Commission resisted repeated attempts to infect them with various strains of *T. gambiense*. Evidently, then, individual differences among a series of animals of the same species may lead to considerable variation in the results obtained by testing them in turn with a given strain of trypanosomes.

However this may be, it seems probable, bearing in mind the results obtained with the other animals, that the failure to superinfect this sheep with a homologous strain of *T. gambiense* was due to immunity acquired during the infection with the original Strain No. II. The fate of this animal when infected with *T. rhodesiense* shows that an immunity against *T. gambiense* is quite ineffectual against the other human trypanosome.

The original strain infecting Goat 341 differs from that just considered by being already non-transmissible when inoculated into the goat. In the goat the strain underwent a very gradual loss of infectivity to tsetse, over a period of one year. At the end of this time, exposure to superinfection with a readily transmissible strain of *T. gambiense* completely failed to produce any demonstrable effect.

Here again, we appear to have an example of the acquisition of immunity, this time seemingly a solid immunity, against *T. gambiense*, as the result of a long-standing infection. Here, too, the immunity against *T. gambiense* had no effect against the Damba trypanosome, a relative of *T. brucei*.

Turning now to the two monkeys. In the case of Monkey 375 there is little to be learnt. At the time of superinfection the strain was still transmissible, and the infection produced no apparent change in the adjustment already established between parasite and host. The behaviour of Monkey 95 is, however, very instructive. When first introduced into this animal, the strain was highly transmissible. The transmissibility index dropped rapidly during the ensuing year, finally reaching zero. Fourteen months after the original infection, the monkey was infected with a descendant of the same strain of trypanosome, and a slight and temporary recrudescence of transmissibility was noted. Again, and this time more rapidly, the index dropped to zero. Some 5 months later, *i.e.* 19 months after the original infection, the animal was infected with a heterologous strain of *T. gambiense*, and immediately a considerable increase in both infectivity and transmissibility occurred. During all this time, and up to the date of my departure on leave 22 months after its first infection, this remarkable little monkey was apparently in excellent health. It is a young adult animal, very active and possessed of a good appetite.

The behaviour of the trypanosome in this monkey suggests a train of interesting reflections. The response of the animal to *T. gambiense* is different from that shown by the domestic ruminants we have just been considering. There has been, as far as one can see, no permanent immunity developed in this monkey. Instead, a condition of tolerance is established in which each new introduction of *T. gambiense* is accompanied by a temporary recrudescence of transmissibility. In the intervals, the animal is non-infective to fly.

Now there seems no reason why the processes revealed by the study of this monkey should not take place in man in areas infected by human trypanosomiasis. Where there is broad contact between man and fly the introduction of a readily transmissible strain will result in the infection of a large proportion of the population. An epidemic results and many die: the contact is reduced and the epidemic declines. Among the survivors who, at this stage, are still in contact with the fly, some will be more exposed to infection than others. Individuals, too, will differ in their resistance and response to infection. Some of these survivors will be infected, and of these some will be carrying non-transmissible strains; these last will be, in ordinary circumstances, non-infectious to fly and, in consequence, harmless to the community. Some of them will probably be tolerant or resistant, and may live a year or more with trypanosomes demonstrable in their system—trypanosomes that are probably non-transmissible.

We know as yet of no absolute immunity in man against declared human trypanosomiasis; so that if all infected people either died or became non-infectious, the parasite would soon disappear—unless, of course, some true reservoir of the human trypanosomes, other than man himself, exists, of which there is no epidemiological evidence.

But the liability to reinfection, or rather superinfection, increases the trypanosome's chance of survival. We must suppose that these more or less resistant human cases, whose strains have become non-transmissible, will once again become infectious when bitten by an infective fly. In this way they can serve as a reservoir of the virus, enabling it to persist more or less sporadically, *in a transmissible form*, until conditions more favourable to its spread recur.

The history of Monkey 95 suggests that the process of revivification may occur several times in the same subject. A single individual, who, like Monkey 95, possesses tolerance of the trypanosome, may thus keep alight a focus of infection, repeatedly infecting fly, and himself receiving fresh transmissible strains into his system. In this way the disease may persist for years in mild endemic or sporadic form, to spread again with epidemic force when famine or some economic pressure restores the breadth of contact necessary for the free propagation of the trypanosome.

In the case of *T. rhodesiense*, its greater pathogenicity lessens the probability of the development of non-transmissible strains in man. Prof. Kleine's recent work with the Commission has, however, brought to light the existence of strains of *T. rhodesiense* that resemble very closely the *T. gambiense* type.

Possibly some of these more chronic strains of *T. rhodesiense* will be found to be non-transmissible. The difference between *T. gambiense* and *T. rhodesiense*, judged from the standpoint of the immunity tests we have been considering, is very striking. Prof. Kleine's latest view on the relationship of these two trypanosomes (Kleine, 1928) is that *T. rhodesiense* is merely *T. gambiense* introduced into a non-immune community. This theory, as applied to the Mwanza *rhodesiense*-area in Tanganyika Territory, necessarily supposes a fairly rapid modification of the introduced strains of *T. gambiense* into the *rhodesiense* variant. It is therefore significant to note that immunity from *T. gambiense* confers no protection whatever against *T. rhodesiense*.

If, on the other hand, *T. gambiense* is a derivative from the *brucei* stock, which, in ages past, became a specialised parasite of man in *palpalis* (and probably *tachinoides*) areas, then it is not surprising that the antigenic values of the two trypanosomes are widely different.

To a believer in this latter view, a typical "mild" *T. gambiense* strain represents the best degree of adjustment yet attained by any trypanosome to a particular and highly specialised host, man. The most finished example of this adjustment is probably to be found in the human trypanosome of the Eket region of Nigeria. Any species of trypanosome, when constrained to utilise man as a definitive vertebrate host, tends, slowly or rapidly, to evolve this solution of its difficulties. Is the procedure ever reversed, and does *T. gambiense, while still dependent on and utilising man*, ever assume the virulent attributes of *T. brucei*? Doubtless we shall one day be able to supply an answer to this query.

At all events Prof. Kleine's view has the advantage of serving as a working hypothesis for the epidemiologist. Observation in *T. gambiense* areas should supply proof whether he is right. It is much more difficult to test definitely the other view, namely, that *T. rhodesiense* is derived directly from *T. brucei*; that successful adaptation of *T. rhodesiense* to man entails inevitably the assumption, sooner or later, of the mild characteristics now ascribed to *T. gambiense*; and finally, that complete dissociation of *T. gambiense* from man, and the substitution, in place of man and *G. palpalis*, of ruminant game animals, with their attendant species of tsetse, will lead to the resumption by the trypanosome of the "*brucei*" characteristics.

III. PROVISIONAL CONCLUSIONS.

I. Natural immunity against *T. gambiense*, as exhibited by sheep and goats, varies from complete immunity to a degree of susceptibility in which the trypanosome may be a contributory, or even, possibly, the direct cause of death. This last contingency is, however, beyond doubt exceedingly rare. Between these extremes we find a peculiar partial immunity which operates solely on the transmissibility of the trypanosome by tsetse, abolishing this property but leaving the strain still capable of producing gut-infections in tsetse and still demonstrable in the peripheral blood.

It is as yet impossible to state whether this loss of transmissibility is attended by any coincident increase in the general resistance of the mammal against the trypanosome infection.

II. Immunity can be *acquired* by sheep and goats against *T. gambiense*. The earliest manifestation of the development of acquired immunity is the disappearance of the transmissibility of the strain by tsetse.

The development of immunity may be arrested at this stage for a considerable time, the strain meanwhile retaining the power to develop in the gut of the fly; or it may continue until the animal is both sterilised of an existing infection and immunised against subsequent infection with the same species of trypanosome. Once established, absolute immunity may persist for at least six months.

When once the first stage of immunisation is reached by an animal, all strains of the same species of trypanosome subsequently introduced into its blood will produce non-transmissible infections. In other words, the animal, though still infected, has been rendered non-infective for at all events a considerable time.

III. Certain individual animals that are susceptible to infection, attain, instead of the partial immunity that we have just described, a condition of equilibrium with the trypanosome. The parasite then causes no inconvenience to its host, and retains its transmissibility by tsetse for a considerable time (at least 15 months).

This state of affairs resembles that described in certain species of antelope infected with *T. gambiense*. We do not yet know whether such a strain preserves its power of infecting man throughout its long sojourn in ruminant blood.

IV. In the case of one of the two monkeys studied, a different type of partial immunity was observed. The transmissibility of the original infecting strain disappeared. Subsequent infection with a transmissible homologous strain temporarily restored the transmissibility in diminished intensity. A third, and this time heterologous transmissible strain then caused a striking increase in the transmissibility. The monkey remained all this time apparently in good condition.

Here is a phenomenon without exact parallel in sheep and goats. This animal had apparently developed a gradually increasing resistance to the trypanosome, but the poise between host and parasite was much less perfect than that attained in Sheep 161.

Monkeys are much more susceptible to the pathogenic action of *T. gambiense* than are ruminants. So, also, is man. It is very seldom that a monkey succeeds against *T. gambiense* as No. 95 has done, but in the case of man many instances are known of prolonged survival after infection by this trypanosome.

What we have noted in the case of Monkey 95 may thus easily occur in man, in regions where *T. gambiense* exists in nature. It is possible, particularly

in certain regions of Western Africa where human trypanosomiasis is found as a very chronic affection endemic for many years, that there exists in man a condition of equilibrium between parasite and host similar to that obtaining in certain ruminant animals.

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THE GAPE-WORM (*SYNGAMUS TRACHEA* MONTAGU) IN ROOKS (*CORVUS FRUGILEGUS* L.).

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During the early part of 1928, a census of the rookeries in the Oxford district was carried out by the Oxford Bird Census organised by Mr E. M. Nicholson. This work raised a number of problems, of which one of the most important was that of natural checks to the numbers of rooks. Although shooting of the young rooks is one of the greatest checks on their population, Mr Nicholson's observations showed that there must also be a mortality amongst the nestlings. Lewis (1925, 1926 *b*) has shown that gape-worms are common in starlings (*Sturnus vulgaris*), and it seemed possible to us that this parasite might also be sufficiently common in rooks to cause deaths among the young birds. The gape-worm had already been recorded from the rook by Lewis (1926 *a*) and Baylis (1928), so that the possibility seemed well worth investigating.

Rooks (41 birds) from the Oxford district were examined during May 1928, and the degree of infestation with gape-worms was found to be surprisingly high. The analysis of these examinations is given below. Dr H. A. Baylis, of the British Museum of Natural History, has very kindly examined some specimens of the worms, and informs us that they are identical in structure with *Syngamus trachea* Montagu (= *S. trachealis* v. Siebold), the species which causes epidemics in poultry. The worms in the rook were, however, rather smaller than usual. To prove the physiological identity, it would be necessary to infect chickens fatally with the rook gape-worm (as has been done by Leiper (1926) in the case of the *Syngamus* from starlings); but it seems, at any rate, probable that these worms from the rooks are actually *S. trachea*.

Old Rooks.

Locality	Total number examined	Number infected with *Syngamus*
Chislehampton	4	2
Nuneham Courtney	1	1
Culham...	1	0
Hardwick	1	0
Swinford Bridge	1	1
Total... ...	8	4

Young Rooks of 1928.

Chislehampton	24	22
Swinford Bridge	8	8
(Not noted)	1	1
Total... ...	33	31

Although the figures are small, they show that a high percentage (94 per cent.) of young rooks may harbour *Syngamus*. All the young birds had just learnt to fly; most of them were infested fairly heavily with gape-worms, which were found living in the larynx, trachea, syrinx, and sometimes in the bronchi. In one case over forty pairs of worms were counted (the males and females live joined together). A large proportion of the other young birds must have been carrying quite as many, although actual counts were not made. Only eight adult rooks were examined, and of these four contained gape-worms in the trachea. But it is noteworthy that in the case of two of the infected birds, only one worm pair was found—a state of affairs never encountered in the young rooks. This fits in with the idea that the rooks become infected when they are young, and that the gape-worms gradually disappear as the birds get older (just as in the case of poultry).

Another point which is of some importance is that the adult rook is capable of carrying gape-worms (in some cases abundantly), and therefore can be considered as a possible carrier and reservoir of the parasite. But, at the same time, it is clear that a much wider survey of rook populations in this and other districts is necessary, before any conclusions can be drawn about the economic status of rooks as gape-worm carriers.

The young rooks undoubtedly suffer from some mortality in the nest before they learn to fly, and in view of the results we have given, and of the analogy with poultry, it seems very likely that they may die of gape-worm infection. In the cases examined, the number of worms present would hardly have sufficed to impede respiration, but the loss of blood may have been important. All the worms were bright scarlet in colour; and a specimen which was sectioned in its natural position in the windpipe (this being done through the kindness of Dr H. M. Carleton of the Department of Physiology), was found to have its mouth firmly imbedded in the lower layers of tissue in the tracheal wall.

The investigation of rook gape-worms has two practical bearings: (*a*) the possibility of rooks introducing the parasite into poultry farms; (*b*) the possibility of gape-worms being one of the main checks on the numbers of rooks, thereby indirectly benefiting agriculture. Further investigation is obviously desirable.

Other wild birds besides the rook have been found to carry *Syngamus trachea*. It has been recorded by Lewis (1926 *a*, 1927), and Baylis (1928) from the pheasant, thrush, starling, magpie, jay, and jackdaw; while a different species, *S. merulae* Baylis, occurs in the blackbird (Baylis, 1926). Lewis (1926 *b*) found that 169 out of 482 starlings (= 35 per cent.) in the Aberystwyth area were carrying *S. trachea*. It would be of interest to know whether the rate of infection is higher in young than in old birds.

We have to thank Prof. E. S. Goodrich for the facilities he has given us for our work in Oxford. We are also indebted to Dr H. A. Baylis, Dr H. M.

Carleton, Mr E. M. Nicholson, and Mr A. D. Middleton, who have helped us in the investigation.

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